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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. 1.53 (c).

Docket Number	290.00500161	Type a plus sign (+) inside this box >	+

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TITLE OF THE INVENTION (280 characters max)

PSEUDOTYPED RETROVIRUSES

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ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification	Number of Pages	104		Small Entity Statement
	Drawing(s)	Number of Sheets			Other (specify)

METHOD OF PAYMENT (check one)

X	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT	($) 160
X	The Commissioner is hereby authorized to charge any additional required fees or credit overpayment to Deposit Account Number: 13-4895		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government:

X No.

____ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date March 27, 2003

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____ Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

jc682 U.S. PTO
03/27/03

03-28-03 [redacted]070-0327 & 1/P10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Sanders et al.

Docket No.: 290.00500161

Title: PSEUDOTYPED RETROVIRUSES

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60/458070

Assistant Commissioner for Patents
ATTN: BOX PROVISIONAL APPLICATION
Washington, D.C. 20231

We are transmitting the following documents along with this Transmittal Sheet (which is submitted in triplicate):

X PROVISIONAL PATENT APPLICATION including:
- X Specification (104 consecutively numbered pgs, including 0 claims);
- ___ Drawings (___ figures on ___ sheets);
- ___ Photographs (___ sheets);
- X Provisional Application Cover Sheet (1 pg).

X A check in the amount of $ 160 to pay the provisional application filing fee.
___ Verified statement(s) establishing small entity status of this application under 37 C.F.R. 1.9 and 1.27 is/are enclosed.
X An itemized return postcard.
___ An Assignment of the invention to _____ and Recordation Form Cover Sheet (___ pgs).
___ A check in the amount of $40.00 to cover the Assignment Recording Fee.
___ Computer readable form of "Sequence Listing." Applicants state that the paper copy form of the "Sequence Listing" section of the present application, and the computer readable form submitted herewith, are the same.
X Other: Application Data Sheet (3 pgs.).

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(PROVISIONAL TRANSMITTAL UNDER RULE 1.10)

[redacted]

APPLICATION DATA SHEET

APPLICATION INFORMATION

Application Type::	PROVISIONAL
Subject Matter::	UTILITY
CD-ROM or CD-R?::	NONE
Title::	PSEUDOTYPED RETROVIRUSES
Attorney Docket Number::	290.00500161

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PSEUDOTYPED RETROVIRUSES

SUMMARY

We have constructed a plasmid (derived from pcDNA3.1) that expresses the Ebola virus (Zaire strain) glycoprotein with its *O*-gylcosylation region deleted (amino acids 309-489) under the control of the cytomegalovirus promoter. We have transfected this plasmid into human cell lines that have been transfected with genes encoding the Moloney murine leukemia virus gag and pol genes. One of these cell lines had been transfected in addition by the plasmid MFG.S-GFP, which expresses a transcript encoding a recombinant Mo-MuLV genome bearing a gene encoding the Aequorea victoria green fluorescent protein. Recombinant pseudotyped retrovirus recovered from the supernatant medium of such cells has been incubated with many different cell lines and has been shown to be capable of introducing the gene encoding the green fluorescent protein into them. These recombinant modified Ebola glycoprotein-pseudotyped retroviruses have substantially improved titers that make *in vivo* gene transfer and gene therapy experiments with such viruses feasible for the first time.

Ebola virus infects a broad spectrum of mammalian hosts; the cells we have constructed will, therefore, also be useful for the introduction of genes into many different cell types. Ebola glycoprotein-pseudotyped retroviruses and lentiviruses have specificity in particular for transducing airway epithelia cells from the apical surface and therefore hold promise for reagents for gene therapy for diseases of the lung such as cystic fibrosis. Through elimination of the *O*-glycosylation region of the Ebola glycoprotein, we have improved expression and consequently virus incorporation of the glycoprotein into recombinant retroviruses. This makes achieving useful titers of Ebola glycoprotein-pseudotyped retroviruses and lentiviruses possible and also increases the safety of these viruses.

PSEUDOTYPED RETROVIRUSES

FIELD OF THE INVENTION

This invention relates generally to pseudotyped viruses and methods of use of the viruses. Specifically, the invention relates to retroviruses or lentiviruses pseudotyped with glycoproteins in which an O-glycosylation domain has been deleted and use of these viruses for gene transfer and gene therapy.

BACKGROUND OF THE INVENTION

Gene therapy is one of the fastest growing areas in experimental medicine. However, most studies are only Phase I or Phase II clinical studies designed mainly to evaluate the toxicity of the viral vectors and constructs being used. A major drawback has been the design of vectors that are both safe and efficacious. Recent efforts in the field have been directed toward the use of retroviral vectors and viral vectors pseudotyped with glycoproteins from highly virulent viruses such as filoviruses.

Retroviruses are ribonucleic acid (RNA) viruses that include an RNA genome enclosed within a viral capsid wherein the capsid is surrounded by an envelope, or lipid bilayer. Glycoproteins present in the lipid bilayer interact with receptors on the surface of various host cells and allow the retroviruses to enter the host cell. Once in the cell, the retroviruses reverse transcribe the RNA of the viral genome into a double–stranded DNA and incorporate the DNA into the cellular genome as a provirus. Gene products from the integrated foreign DNA may then be produced so that progeny viral particles may be assembled. As retroviruses can be modified to carry exogenous nucleotide sequences of interest, such recombinant retroviruses have a variety of uses. For

example, such recombinant retroviruses are important in introducing desired exogenous sequences into a cell, so that relatively high levels of the protein encoded by the sequences my be produced. However, use of such recombinant retroviruses has several drawbacks.

One such drawback is that retroviruses do not have a broad host range. Efforts at increasing the host range of retroviruses have included substituting the envelope glycoproteins of the retrovirus with that of a different virus, thus forming a pseudotyped retrovirus. The pseudotyped retrovirus advantageously has the host range of the different virus. However, some retroviruses have been pseudotyped with viral glycoproteins that are toxic to cells, so the cells can only produce the virus for a limited time. Furthermore, in many cases, the pseudotyped retroviruses can not be stably produced and may not be produced at a high titer. Stable cell lines have been developed to overcome the toxicity problems and to stably produce such pseudotyped retroviruses. However, there still exists a need for pseudotyped retroviruses that will allow for the production of high titers that would be required for routine gene transfer and/or gene therapy.

Thus it would be desirable to have a pseudotyped retrovirus that is not toxic to cells and produces high titers of a competent virus. It would also be desirable to have a cell line to produce such retroviruses. Methods for using such a virus would also be desirable.

SUMMARY OF THE INVENTION

It has been discovered that deleting the O-glycosylation domain of a viral glycoprotein of a pseudotyped retrovirus allows for stable production of the pseudotyped virus from cell lines. Pseudotyped viruses having viral glycoproteins with a deleted O-glycosylation domain were produced in higher titer than those with wild-type viral

glycoproteins and were also more efficient in transfecting target cells. Accordingly, one aspect of the invention provides pseudotyped retroviruses, including a retroviral capsid, a lipid bilayer surrounding the retroviral capsid an at least one viral glycoprotein disposed in the lipid bilayer in which the O-glycosylation domain has been deleted, in whole or part. In one embodiment, the viral glycoprotein is a filoviral glycoprotein such as, but not limited to, Ebola virus or Marburg virus glycoprotein.

In another aspect of the invention, cells for producing pseudotyped retroviruses having a viral glycoprotein in which the O-glycosylation domain is deleted, in whole or part. Accordingly, the present invention provides eukaryotic cells that include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein in which the O-glycosylation domain of the glycoprotein has been deleted. Preferably, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as, for example, a Marburg virus or Ebola virus glycoprotein. In a preferred form of the invention, the cells stably produce inventive pseudotyped retroviruses.

In a further aspect of the invention, methods for introducing nucleotide sequences into a cell using the pseudotyped viruses of the present invention. The viruses can be used to introduce a nucleotide sequence into a cell *in vitro* or *in vivo*. Preferably, the viruses of the present invention are used for *in vivo* introduction of a nucleotide sequence into a wide range of cell types.

In yet another aspect of the invention methods are provided for producing high titers of pseudotyped retroviruses. The methods involve introducing into a cell nucleotide sequences that encode for a viral glycoprotein in which the O-glycosylation domain is mutated. Also present in the cells are nucleotide sequences that encode for

other proteins necessary to produce a pseudotyped retrovirus in high titers and being more efficient in transfecting target cells.

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and by referencing the following drawings in which:

Figure 1 is schematic representation of the Ebola virus glycoprotein showing the GP_1 and GP_2 subunits of the glycoprotein drawn to scale with residue numbers indicated below the diagram;

Figure 2 is a western blot showing the expression and incorporation of the Δ308-489 Ebola GP into pseudotyped retroviruses; and

Figure 3 is a western blot showing the extent of O-glycosylation of the Δ308-489 Ebola GP incorporated into pseudotyped retroviruses.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that deleting the O-glycosylation domain of a viral glycoprotein of a pseudotyped retrovirus allows for stable production of the pseudotyped virus from cell lines. Pseudotyped viruses having viral glycoproteins with a deleted O-glycosylation domain were produced in higher titer than those with wild-type viral glycoproteins and were also more efficient in transfecting target cells. Accordingly, one aspect of the invention provides pseudotyped retroviruses, including a retroviral capsid, a lipid bilayer surrounding the retroviral capsid an at least one viral glycoprotein

disposed in the lipid bilayer in which the O-glycosylation domain has been deleted, in whole or part. In one embodiment; the viral glycoprotein is a filoviral glycoprotein such as, but not limited to, Ebola virus or Marburg virus glycoprotein.

It has been discovered that eukaryotic cells may be constructed that stably produce pseudotype retroviruses having a viral glycoprotein disposed in their lipid bilayer wherein the O-glycosylation domain of the viral glycoprotein has been deleted in whole or part. It was unexpectedly found that deletion of the O-glycosylation domain a filoviral glycoprotein resulted in increased expression and transduction of the glycoprotein. Elimination of the domain produces enhanced glycoprotein processing and incorporation into retroviral particles, allowing for stable production of the pseudotyped viruses. Furthermore, the levels of transduction are significantly higher by the pseudotyped retroviruses having glycoproteins with mutated O-glycosylation domains as compared to wild-type pseudotyped retroviruses.

The present invention contemplates that the O-glycosylation region of the glycoprotein is deleted in whole or part. While not wishing to be bound by theory, it is thought that deletion of the O-glycosylation region allows for increased expression and transduction by by-passing the post-translation glycosylation step. This step may be rate limiting and glycosylation of high amounts of viral glycoproteins may be toxic to the cells. Therefore, deletion of the O-glycosylation domain results in stable production of pseudotyped retroviruses with increased expression and transduction of the mutated glycoprotein. By "stable production" or "stably produced", it is meant that the cells will produce pseudotyped retroviruses indefinitely (i.e., during the life span of the cell). The viral glycoprotein may be any viral glycoprotein having an O-glycosylation region. The O-glycosylation region is identified by being rich in proline, serine and threonine residues. Filoviral glycoproteins, such as those of Ebola and Marburg, have O-glycosylation regions. By way of non-limiting example, the O-glycosylation domain for

the Ebola glycoprotein is from about nucleotides 309-489 of SEQ. ID. NO. 1.

It will be appreciated by the skilled artisan that the O-glycosylation domain can be deleted either in whole or part. This can be accomplished, for example, by removing the part of the sequence encoding the domain. Alternatively, the nucleotide sequence can be modified to replace the codons for serine and threonine, the amino acids that are O-glycosylated, with codons for conservative amino acid substitutions which will not be O-glycosylated.

As discussed above, one aspect of the invention provides eukaryotic cells, forming inventive eukaryotic cell lines, having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at least one viral glycoprotein, such as a filoviral glycoprotein, in which the O-glycosylation domain of the glycoprotein has been deleted or modified. The term "eukaryotic cell line" as used herein is intended to refer to eukaryotic cells that are grown *in vitro*. The term "nucleotide sequence", as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

In forming a cell that produces an inventive pseudotyped retrovirus, a wide variety of cells may be selected. Eukaryotic cells are preferred, whereas mammalian cells are more preferred, and include human, simian canine, feline, equine and rodent cells. Human cells are most preferred. It is further preferred that the cell be able to reproduce indefinitely, and is therefore immortal. Examples of cells that may be

advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby canine kidney cells and human embryonic 293T cells. However, highly transfectable cells, such as human embryonic kidney 293T cells, are preferred. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

The retroviral *gag, pro* and *pol* nucleotide sequences, and other retroviral nucleotide sequences for forming the specified pseudotyped retroviruses, may be obtained from a wide variety of genera in the family Retroviridae, including, for example, Oncoviruses, including Oncovirus A, B, C and D, lentiviruses and spumavirus F. Such sequences are preferably obtained from the Moloney murine leukemia virus (MMLV; in the genus Oncovirus C). Such sequences are well known in the art. For example, nucleotide sequences encoding MMLV gag, pro and pol may be found in Bereven et al., *Cell* (1981) 27:97-108. Most preferably, such sequences are obtained from lentiviruses. Unlike most retroviruses, lentiviruses have the capacity to integrate the genetic material they carry into the chromosomes of non-dividing cells as well as dividing cells. Therefore, lentiviral nucleotide sequences encoding proteins that allow for chromosomal integration of virally transported nucleic acid in non-dividing cells are advantageously employed, as the host range of the pseudotyped retroviruses will be broadened.

The above-described retroviruses are readily publicly available from the American Type Culture Collection (ATCC) and the desired nucleotide sequences may be obtained from these retroviruses by methods known to the skilled artisan. For example, the nucleotide sequences may be obtained by recombinant DNA technology. Briefly, viral DNA libraries may be constructed and the nucleotide sequences may be obtained by standard nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using appropriate probes or primers. Alternatively, supernatant medium from cells

infected with the respective virus can be isolated and the desired retroviral nucleotide sequences may be amplified by PCR. Such vectors may also be constructed by other methods known to the art.

It is preferred that the *gag*, *pro* and *pol* nucleotide sequences are contiguous to each other as found in native retroviral genomes, such as in the order 5'-gag-pro-pol-3'. It is further preferred that these retroviral nucleotide sequences are chromosomally-integrated into the cellular genome. Furthermore, the gag-pro-pol nucleotide sequences are operably linked at the 5' end of the *gag* nucleotide sequence to a promoter sequence, so that transcription of the sequences may be achieved.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region. Typically, the promoter element is generally upstream (i.e., at the 5 end) of the nucleic acid coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by activating elements known in the art, so that production of the protein encoded by the specified nucleic acid sequence may be regulated as desired, It is well within the purview of a person skilled in the art to select

and use an appropriate promoter in accordance with the present invention. For example, the promoters that may be advantageously present in the cell, 5' to the gag-pro-pol sequences, include rat actin promoter and the MMLV promoter. Furthermore, the cytomegalovirus promoter has been found to be an excellent promoter in the inventive system.

Other regulatory elements, such as enhancer sequences, which is cooperate with the promoter and transcriptional start site to achieve transcription of the nucleic acid insert coding sequence, may also be present in the cell 5' to the nucleotide sequences that encode retroviral proteins. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

A wide variety of viral glycoproteins may be advantageously present in the inventive cells of the present invention, especially viral glycoproteins having an O-glycosylation domain and which are necessary for attachment of the virus to a target cell and penetration of the virus into the cytoplasm of the cell, as well as viral glycoproteins necessary for maturation of the glycoproteins necessary for attachment and penetration of the virus. For example, the cells described above may include nucleotide sequences filoviral glycoproteins. Examples of such viruses include Ebola virus (including Ebola Zaire, Ebola Reston and Ebola Sudan sequences which are chromosomally-integrated), and Marburg virus. These nucleotide sequences may be obtained by methods known in the art. For example, nucleotide sequences encoding particular glycoproteins may be isolated and cloned into plasmids by standard techniques, and the nucleotide sequence may then be amplified by FOR using the appropriate primers.

In one embodiment, the cells include nucleotide sequences encoding glycoproteins from a filovirus. Such filoviruses also exhibit a broad host range. A wide variety of nucleotide sequences that encode filoviral glycoproteins may be used to produce the inventive cells of the present invention. For example, nucleotide sequences

encoding glycoproteins from the Marburg and Ebola virus (in the family Filoviridae and, including, for example, Ebola-Zaire and Ebola-Reston) may be introduced into the cells described above to produce a pseudotyped retrovirus. SEQ ID 2 shows the Ebola Zaire glycoprotein-encoding sequence and SEQ ID 3 shows the Marburg virus glycoprotein-encoding sequence. The nucleotide sequences encoding the filoviral glycoproteins may be obtained as described in Sanchez et al. (1993) *Virus Res.* 29 (3):215-240 and Will et al., (1993) *J. Virot* 67:1203-1210. Moreover, such sequences may be obtained by other methods known to those skilled in the art.

It is expected that other viruses not specifically mentioned above and having glycoproteins of similar structure to the filoviral glycoproteins may be advantageously used in the present invention.

In another embodiment of the present invention, the eukaryotic cells described above may include another nucleotide sequence that encodes a desired protein so that they may produce pseudotyped retroviruses having an RNA genome including such desired nucleotide sequences. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleotide sequence may be introduced into the cellular genome in a variety of ways known to the skilled artisan. For example, defective retroviruses (i.e., those which do not have the capability to produce all of the viral proteins necessary for production of a retrovirus having the ability to infect a cell and produce progeny viruses) may be constructed to include such a sequence in their RNA genome and can then transduce a cell. Alternatively, plasmid vectors may be used to introduce the nucleotide sequence, preferably DNA, encoding the desired protein. In either case, the vector typically

includes nucleotide sequences necessary for production of the pseudotyped retrovirus. For example, the RNA sequence in the viral genome is flanked on the 5' end by a splice acceptor site and a splice donor site followed by a sequence necessary for packing of the viral genome (such as a psi sequence) and a long terminal repeat (LTR), all as known in the art. The 3' end of the RNA sequence may be flanked on its 3' end with a polypurine tract followed by another LTR, further as known to the skilled artisan. The vectors may include other nucleotide sequences known to the art that are necessary for transduction.

In one preferred form, the desired protein may be one that allows entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light; or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleotide sequences include the nucleotide sequence encoding the *Aequorea victoria* green fluorescent protein [GFP; nucleotide sequences listed in Prasher et al., (1992) *Gene* 111:229] and the LacZ gene (produces 3-galactosidase), both of which are well known in the art and may be obtained commercially.

Another aspect of the invention provides methods of forming eukaryotic cells for producing pseudotyped retroviruses. The method includes introducing into the cells described above the nucleotide sequences described above, i.e., those encoding the retroviral Gag, Pro and Pol polypeptides, and those encoding a filoviral glycoprotein in which the O-glycosylation domain is deleted or mutated, in part or whole, into the cell.

The nucleotide sequences may be introduced into the desired cell utilizing a variety of vectors known to the skilled artisan. For example, plasmid vectors, cosmid vectors, and other viral vectors, such as retroviral vectors, may be used. It is preferred that the nucleotide sequences encoding the Gag, Pro and Pol polypeptides are on a separate vector than the nucleotide sequences encoding the viral glycoproteins.

In one mode of practicing the invention, plasmid vectors are advantageously used to introduce, or transfect, the nucleotide sequences into the selected cell. A wide variety of plasmid vectors may be used, including pTRE, pCMV-Script and pcDNA3, although pcDNA3 is a preferred vector. The *gag, pro* and *pol* nucleotide sequences are preferably on the same plasmid, and, as discussed above, are preferably contiguous to each other. However, the skilled artisan is aware that other spatial configurations of the nucleotide sequences may be utilized when constructing the plasmids. The vector also preferably includes a promoter 5' to, or upstream from, the *gag* nucleotide sequence. The vectors may further include other regulatory elements, such as enhancer sequences, as discussed above.

The nucleotide sequences encoding the viral glycoproteins are preferably on a separate plasmid, or other vector, than the *gag, pro* and *pol* nucleotide sequences. The viral glycoprotein encoding sequences, such as the sequences encoding the filoviral glycoproteins are also preferably operably linked to a promoter sequence described above. It is also understood that the nucleotide sequences encoding at least two different viral glycoproteins may be arranged on a vector such that the nucleotide sequences encoding one of the glycoproteins are present on one vector and the sequences encoding the other glycoprotein are present on a different vector. It is preferred, however, that such sequences are on the same vector, and preferably contiguous to each other so they will be transcribed utilizing the same promoter. In one preferred form of the invention, the promoter sequence is a cytomegalovirus promoter sequence. Plasmids, or other vectors carrying the nucleotide sequences encoding the viral glycoproteins, may also include other regulatory elements, such as enhancers, as described above.

The vectors may be introduced into the cells in a variety of ways known to the skilled artisan, for example, discussed in Current Protocols in *Molecular Biology,* John

Wiley and Sons, edited by Ausubel et al. (1988) and Maniatis, et al., *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory (1989). For example, vectors may be transfected into a cell by a calcium phosphate precipitation method. Other methods for introduction of the vectors include, for example, electroporation and lipofection.

In many cases, one may wish to quickly visually detect those cells which have taken up a vector and that produce a specified protein from the vector. Visually detectable components, or markers, include the *Aequorea victoria* green fluorescent protein as discussed above. When forming a cell that includes a visually detectable component, or marker, the nucleotide sequences encoding the marker may also be introduced into the cell as described above. Cells that have taken up the vector and express the nucleotide sequences encoding a protein may be identified and separated from cells that do not express the sequences by a fluorescence-activated cell sorting procedure as known in the art. A visually detectable marker may also be formed from reaction of β-galactosidase (produced by the LacZ gene) with a substrate, such as X-gal.

In a third aspect of the present invention, pseudotyped retroviruses that include viral glycoproteins in which the O-glycosylation domain has been deleted or modified, in whole or part, (as discussed above) disposed in their lipid bilayer are provided. In a preferred embodiment, the glycoprotein is a filoviral glycoprotein.

In one embodiment, such pseudotyped retroviruses include a core is RNA genome that is surrounded by, or enclosed within, a viral capsid. The genome preferably includes a nucleotide sequence encoding a protein selected to be subsequently produced by a cell. The genome further includes other nucleotide sequences for formation of the pseudotyped retrovirus, such as 5' and 3' LTR sequences that are operably linked to the nucleotide sequence encoding the desired protein as described above. Reverse transcriptase and integrase are also enclosed within the capsid, which gives the retrovirus the ability to incorporate a gene encoding a desired protein into a

genome of a cell after the retrovirus contacts, or is incubated with, the cell. For example, the pseudotyped retrovirus may be used to incorporate a gene encoding an enzyme in a host cell that is incapable of producing the enzyme, or produces a non-functional enzyme as discussed above. Other sequences known to the art that are useful for transducing genes may also be present in the RNA genome.

The pseudotyped retrovirus may include other proteins, in addition to integrase, that aid its stable integration into the chromosomes of a target cell. For example, with respect to a lentivirus, the pseudotyped retrovirus may include proteins such as vpr, vif and vpu.

In yet other preferred embodiments, the pseudotyped retrovirus may include a nucleotide sequence encoding a visually detectable component, or marker, such as *Aequorea victoria* green fluorescent protein as discussed above. Such a retrovirus may be advantageously used in a method of determining viral entry into a cell discussed above. Moreover, such a virus is advantageously used in the methods of the present invention to ensure that the pseudotyped retroviruses that are formed are replication incompetent (i.e., do not have all the sequences necessary in their viral genome to produce progeny retroviruses). For example, supernatant isolated from cells transduced by the vectors and contacted with a test cell should not result in localization of the fluorescent protein in the test cell.

In a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided. In one embodiment, the method includes contacting, or transducing, a cell permissive for filoviral entry, with a retrovirus that has been pseudotyped with a filoviral glycoprotein as described above that includes the desired nucleotide sequence in its genome. When the nucleotide sequences encode a desired protein, the cell is selected so that it also preferably allows expression of the selected nucleotide sequence. The level of transduction may be obtained by assaying methods

known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences.

Reference will now be made to specific examples illustrating the compositions and methods above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention in intended thereby.

EXAMPLE

MATERIALS AND METHODS

Cell lines and culture conditions. The human kidney cell line 293 (ATCC Number CRL-1573), the mouse embryo cell line NIH 3T3 (CRL-1658), and the 293T-derived ϕNX (second generation retroviral packaging cells) (11, 18, 26) and gpnlslacZ cell lines were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 units Penicillin G, and 100 µg/ml streptomycin sulfate, with or without 0.25 µg/ml amphotericin B (growth medium). The gpnlslacZ cells produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S-nlslacZ, a retroviral vector encoding a nuclear localizing β-galactosidase (25).

Plasmids and site-directed mutagenesis. A modified version of the plasmid pTM1 was used in transient expression studies of GP sequences using a vaccinia virus-T7 RNA polymerase (VV-T7) system (5). The pTM1 vector was modified to remove an ATG codon (within an *Nco*I site) at the beginning of the multiple cloning site by *Nco*I digestion, mungbean nuclease treatment, and ligation of the blunt-ended DNA. This vector,

pTM1(ΔNcoI), was used to subclone the entire Ebola virus GP open reading frame (ORF). The GP ORF was cleaved from the plasmid pGEM-EMGP1 (21) by digestion with *Bam*HI and *Dra*I, and the fragment isolated and directionally ligated into the pTM1(ΔNcoI) vector cleaved with *Bam*HI and *Stu*I. The resulting clone, pTM1(ΔNcoI)-GP, was used as the target DNA for all site-directed mutagenesis reactions. This clone encodes a GP sequence that differs from the wild-type amino acid sequence in a single residue within the membrane spanning sequence (I662V), and for comparative purposes will be referred to as "wild-type sequence". This mutation is present in the original pGEM3Zf(-)-GP clone, but does not appear to affect the processing or function of the GP. GP residue numbering commences with the methionine of the signal sequence and is continuous through the GP_1 and GP_2 sequences.

The GP clone in which the mucin region was deleted (Δ309-489) was generated from two PCR clones linked by an *Xba*I restriction site, which resulted in the replacement of the mucin sequence with two residues (serine-arginine). Mutations in isolated plasmid clones were identified by direct sequencing of mini-prep DNA using dye-terminator cycle sequencing reactions (ABI) analyzed on either an ABI 373 or 377 sequencer. Large-scale preparations for each type of mutated plasmid DNA were made using commercial kits (Promega Corp. or 5 Prime → 3 Prime, Inc.). The DNA was quantified by UV_{260} absorbance readings, and then stored at -70°C until needed. The coding region (*Bam*HI/*Sal*I fragments) from the plasmid pTM1(ΔNcoI)-GP and mutated versions of this DNA were separately ligated into the *Bam*HI/*Xho*I polylinker sites of the vector pcDNA3 (Invitrogen), cloned in *E. coli*, and plasmid DNA isolated for use in pseudotyping studies.

Retrovirus pseudotyping and virus transduction assays. Pseudotyped retrovirus particles consisting of the MuLV cores and the Ebola GP in their envelopes were produced by transfecting wild-type or mutated plasmid DNA into gpnlslacZ cells as

previously described by Sharkey, C.M. et al., *J. Virol.* 75:2653-2659 (2001). Virus transduction of β-galactosidase activity into NIH 3T3 cells was determined as described by Sharkey, C.M. et al., *J. Virol.* 75:2653-2659 (2001). All data presented are the average of the results of at least three experiments.

Immunoblot analysis of Ebola virus glycoprotein expression, processing, and incorporation into pseudotyped retroviruses. Medium from transfected ϕNX cells (11, 18, 26) containing recombinant retroviruses were passed through a 0.45 μm filter and centrifuged through a 30% sucrose cushion at 25,000 RPM in a Beckman 50.2-Ti rotor in a Beckman SS-71 centrifuge. The fluid was aspirated from centrifuge tubes and discarded, and the virus pellet was suspended in 100μl of RIPA buffer (140 mM NaCl, 10 mM Tris HCl pH 8.0, 5 mM EDTA, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS). Cells were treated with lysis buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100), and the cell lysates were then centrifuged in a microcentrifuge at 16,100 x *g* for ten minutes. The proteins in the cell lysate and the suspended viral pellet were each precipitated with a final concentration of 4% TCA for two minutes. The precipitated proteins were then centrifuged at 16,100 x *g* in a microcentrifuge for ten minutes. The supernatant fluid was aspirated and discarded. The pellet was suspended in an equal volume of 1 M Tris[hydroxymethyl]-aminomethane and vortexed vigorously. Proteins whose glycosylation was analyzed were treated sequentially with PNGase F, which removes *N*-linked glycosylation, and with both Sialidase A and Endo-O-glycosidase (ProZyme, Inc.), which together remove O-linked glycosylation, following protocols provided by the supplier. The pellet suspension was then mixed with 1/6 the volume of 300 mM Tris pH 6.8, 60% glycerol (w/v), 4% SDS (w/v), 0.0012% bromophenol blue (w/v), 6% 2-mercaptoethanol (v/v) and boiled for 5 minutes. Equal amounts of proteins as determined by the Bradford assay were separated by SDS-

PAGE (8.5% acrylamide), and electrophoretically blotted onto nitrocellulose membranes. Membrane blots were immersed in reaction buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) containing 1% bovine serum albumin and incubated overnight at 4°C. Blots were incubated in reaction buffer containing a rabbit-anti-Ebola SGP/GP diluted 1:1000 for 1 hr at room temperature, washed 3 times in reaction buffer, and reacted with a Goat anti-rabbit-horseradish peroxidase conjugate (diluted 1:20,000 in reaction buffer) for 30 min at room temperature. Membranes were washed as before, then treated with a commercial chemiluminescent substrate solution (Amersham Pharmacia Biotech), according to the protocols provided by the manufacturer. Specific reactivity to GP was visualized by exposing treated blots to X-ray film.

RESULTS

Pseudotyped retroviruses bearing GPs with altered glycosylation. The role of O-glycosylation of the Ebola virus GP was examined through analysis of the effects of deleting the region of the protein that is O-glycosylated. Remarkably processing and viral incorporation of the Δ309-489 GP was greatly enhanced as shown in Figure 2. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform) , GP_{pre} (the *N*-glycosylated but not O-glycosylated uncleaved glycoform) and deglycosylated GP_0 and GP_{pre} forms of the wild-type GP and of the GP_1, GP_0 and deglycosylated GP_1 and GP_0 forms of the Δ309-489 GP is indicated There was also a corresponding increase of 696 ± 142% in transduction by the Δ309-489 GP pseudotyped viruses as compared to wild type. The absence of an increase in the mobility of the Δ309-489 GP upon sialadase A and endo-O-glycosidase treatment provides confirmation that the region of O-glycosylation of the glycoprotein has been removed (Figure 3). The migration of the mature GP_1 of the wild-type and Δ309-489 GPs is indicated. In this experiment a glycosylated serum protein possessing a mobility intermediate between those of the

wild-type and Δ309-489 GP_1s was detected. The heterogeneous mobility of the PNGaseF-treated proteins is indicative of incomplete removal of *N*-glycosylation.

The effect of deleting the O-glycosylation region of GP_1 (Δ309-489) on expression and transduction were striking. This segment, which is rich in proline, serine, and threonine residues is the most variable among the Ebola GPs. Elimination of this mucin-like domain results in enhanced GP processing and incorporation into retroviral particles (Figure 2) and consequently higher levels of transduction by the pseudotyped retroviruses. It is possible that the wild-type GP is retained in the Golgi apparatus until all of the serines and threonine residues in the mucin region are modified. While not wishing to be bound by theory, it is thought that elimination of this segment may permit more rapid transit through the Golgi apparatus and higher levels of processing to $GP_1$and GP_2 and of cell-surface expression. Increased viral incorporation may also result from a diminution of GP toxicity. It has been reported that the deletion of the O-glycosylation region reduces the cytopathic effects of Ebola virus GP expression (40). It has also been suggested that the expression of high levels of the wild-type Ebola GP might lead to exhaustion of the cellular glycosylation machinery (32), which is consistent with the present results and present interpretation.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

Patent and literature references cited herein are incorporated by reference as if fully set forth.

ABSTRACT

Pseudotyped retroviruses having viral glycoproteins with deleted or mutated O-glycosylation domains are provided. Also provided are methods for making the pseudotyped retroviruses of the present invention. Cells for stably producing the pseudotyped retroviruses or the present invention are also provided.

EXAMPLE

Covalent Modifications of the Ebola Virus Glycoproteins: Implications for Structure, Evolution, and Gene-Transfer Technologies

The roles of the covalent modifications of the Ebola virus glycoprotein (GP) and the significance of the sequence identity between filovirus and avian retrovirus glycoproteins were investigated through biochemical and functional analyses of mutant GPs. The expression and processing of mutant GPs with altered *N*-linked glycosylation, substitutions for conserved cysteine residues, or a deletion in the region of *O*-glycosylation were analyzed, and viral entry capacities were assayed through use of pseudotyped retroviruses. Cys-53 was shown to form the only disulfide bond linking GP_1 to GP_2. We propose a cystine-bridge map for the filovirus glycoproteins based upon our analysis of mutant Ebola virus GPs. The effect of replacement of the conserved cysteines in the membrane-spanning region of GP_2 was found to depend on the nature of the substitution. Mutations in conserved *N*-linked glycosylation sites proved generally, with a few exceptions, innocuous. Deletion of the *O*-glycosylation region increased glycoprotein processing, incorporation into retrovirus particles and viral transduction. Our data support a common evolutionary origin for the glycoproteins of Ebola virus and avian retroviruses and have implications for gene transfer mediated by Ebola virus glycoprotein-pseudotyped retroviruses.

INTRODUCTION

Ebola viruses are a group of enveloped, single-stranded, RNA viruses that, together with Marburg virus, are classified in the Order Mononegavirales and the Family *Filoviridae*. Filoviruses cause a severe hemorrhagic fever disease in human and/or nonhuman primates and are designated as biosafety level 4 agents. These viruses contain a single structural glycoprotein (GP) that form the peplomers that project from the surface of the enveloped, rod-shaped virion (7, 22, 23). The GP of filoviruses are expressed from the GP gene, but the organization of this gene differs dramatically between Ebola and Marburg viruses. The GP of all Marburg virus isolates are encoded in a single open reading frame (ORF), whereas the GP of Ebola viruses are encoded in two frames (0 and -1) that are connected by transcriptional editing that results in an insertion of a single base (22, 29). The primary gene product of the Ebola virus GP gene is a secreted glycoprotein (cleaved to generate SGP and delta-peptide (35)), whereas the structurally important GP is a secondary gene product. The functions of SGP and delta-peptide are not well defined, but biochemical, immunological, and structural studies have provided clearer insights into the role of GP in virus entry and pathogenesis (2, 7, 8, 12, 13, 16, 20, 22, 23, 31, 36, 38). The peplomers covering the surface of Ebola virion are composed of GP trimers anchored in the lipid bilayer by a transmembrane sequence in a type I orientation (21-23). These structures mediate entry of the virion into cells through a process of: 1) binding to receptor molecules, 2) endocytosis of the virion, 3) acidification of the endocytic vesicle, and 4) membrane fusion brought about by acid-induced conformational changes in GP (1, 12, 27, 37, 39).

Processing of GP in the cell leads to the production of various forms as it travels through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane (31). An *N*-glycosylated precursor form GP (GP_{pre}), which is found within the ER, is further processed to a fully glycosylated uncleaved form in the Golgi apparatus (GP_0); trafficking to the Golgi apparatus also leads to the addition of *O*-linked glycans (7, 31). In the trans Golgi GP_0 is cleaved by the convertase furin to generate GP_1 (~130 kDa), whose role appears to involve receptor binding (21), and the transmembrane GP_2 (~24 kDa); these two subunits are linked by disulfide bonding (22,

23, 31). Fig. 1 shows a diagrammatic view of the Zaire species of Ebola virus GP. GP_1 is highly glycosylated with *N*-linked and *O*-linked glycans (7). Glycosylation contributes approximately half of the mass of GP_1, and *O*-glycans confer a mucin-like property to its C-terminus. GP_2 also contains *N*-linked glycans (22, 23, 31) with two predicted *N*-linked sites, but does not appear to contain *O*-linked glycans.

The disulfide bonding that holds the GP_1-GP_2 heterodimer together is predicted to involve the first cysteine of GP_1 (cys-53) and the fifth cysteine from the amino-terminus of GP_2 (cys-609). This prediction is based on sequence and structural similarities of Ebola virus GP to the glycoproteins of the Avian Sarcoma and Leukosis Viruses (ASLVs) (10) and other retroviruses (6, 16, 19, 24, 30) and the fact that the cys-53 residue of SGP is involved in forming the SGP homodimer (23, 34). The intramolecular disulfide bonding of GP_2 has also been predicted based on sequence similarity with the ASLV glycoproteins (10). A putative fusion peptide of 16 hydrophobic and uncharged residues has been identified near the amino-terminus of GP_2, and a synthetic version of this peptide was shown to penetrate and induce fusion of membranes containing phosphatidylinositol (20). The membrane-spanning anchor sequence near the C-terminus of the Ebola virus GP_2 contains two conserved cysteine residues that are palmitoylated (13). X-ray crystallography of recombinant-expressed portions of GP_2 have shown that alpha helices in the sequence form coiled coils, and that these structures are remarkably similar to those of the transmembrane glycoproteins of retroviruses (TM, p41) and influenza viruses (HA2), as well as SNAREs (cellular proteins involved in fusion of transport vesicles) (16, 36).

Recombinant DNA techniques have also been employed to safely perform functional studies of the Ebola GP peplomer through pseudotyping of engineered vesicular stomatitis virus (12, 27) and retroviruses (1, 37-39). Pseudotyped retrovirus particles have been used to demonstrate permissiveness to virus entry of endothelial cells and the relative lack of susceptibility of lymphocyte cell lines to transduction (1, 37, 39). In addition, it has been shown that mutations of the fusion peptide sequence block virus entry (12), and that elimination of furin cleavage during processing of the Ebola virus GP does not prevent virus entry (13, 38).

To further define the effects of the covalent modifications of GP_1 and GP_2 upon their function, we have performed site-directed mutagenesis of plasmid DNA to change specific residues of the encoded proteins. These mutated sequences were then used to study the affects of these changes on processing, disulfide bonding, and virus entry via pseudotyped retrovirus particles. We specifically wished to determine the functional significance of the residues that are conserved between the filovirus and ASLV glycoproteins and the role of the *O*-glycosylation region of the Ebola virus glycoprotein.

MATERIALS AND METHODS

Cell lines and culture conditions. The human kidney cell line 293 (ATCC Number CRL-1573), the mouse embryo cell line NIH 3T3 (CRL-1658), and the 293T-derived ϕNX (second generation retroviral packaging cells) (11, 18, 26) and gpnlslacZ cell lines were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 units Penicillin G, and 100 µg/ml streptomycin sulfate, with or without 0.25 µg/ml amphotericin B (growth medium). The gpnlslacZ cells produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S-nlslacZ, a retroviral vector encoding a nuclear localizing β-galactosidase (25).

Plasmids and site-directed mutagenesis. A modified version of the plasmid pTM1 was used in transient expression studies of GP sequences using a vaccinia virus-T7 RNA polymerase (VV-T7) system (5). The pTM1 vector was modified to remove an ATG codon (within an *Nco*I site) at the beginning of the multiple cloning site by *Nco*I digestion, mungbean nuclease treatment, and ligation of the blunt-ended DNA. This vector, pTM1(Δ*Nco*I), was used to subclone the entire Ebola virus GP open reading frame (ORF). The GP ORF was cleaved from the plasmid pGEM-EMGP1 (21) by digestion with *Bam*HI and *Dra*I, and the fragment isolated and directionally ligated into the pTM1(Δ*Nco*I) vector cleaved with *Bam*HI and *Stu*I. The resulting clone, pTM1(Δ*Nco*I)-GP, was used as the target DNA for all site-directed mutagenesis reactions. This clone encodes a GP sequence that differs from the wild-type amino acid sequence in a single residue within the membrane spanning sequence (I662V), and for comparative purposes will be referred to as "wild-type sequence". This mutation is present in the original pGEM3Zf(-)-GP clone, but does not appear to affect the processing or function of the GP. GP residue numbering commences with the methionine of the signal sequence and is continuous through the GP_1 and GP_2

sequences.

Site-directed mutagenesis targeted conserved cysteines and asparagines in conserved *N*-linked glycosylation sites. Table 1 shows 21 mutant GPs that were used for analysis of GP_1 and GP_2 in the VV-T7 system. Additional mutants (T42D, double substitutions for the GP_1 cysteines (C108G/C135S,C108G/C147S,C121G/C135S, and C121G/C147S), C670A, C672A, and C670A/C672A, and Δ309-489) were also generated, but were used only in the pseudotyping experiments. Mutagenesis of plasmid DNA sequences were performed using commercial kits, either the GeneEditor *in vitro* system (Promega Corp.) or the MORPH system (5 Prime → 3 Prime, Inc.), according to the manufacturer's instructions. Briefly, 5' phophorylated mutagenic primers ranging in length from 25 to 36 nucleotides (mismatches centered in the sequence) were annealed to denatured pTM1(Δ*Nco*I)-GP DNA, primers extended with T4 DNA polymerase, and ends ligated with T4 DNA ligase. Plasmid DNAs with mutations were enriched by specific antibiotic selection (GeneEditor) or digestion with *Dpn*I prior to transformation (MORPH). Mutant DNAs were used to transform *Escherichia coli* (*E. coli*) mismatch repair mutants (BMH 71-18 or MORPH *mut*S cells), then mini-prep DNA was isolated (5 Prime → 3 Prime, Inc.) and used in second round transformation of JM109 *E. coli* to isolate mutated DNA strands. The GP clone in which the mucin region was deleted (Δ309-489) was generated from two PCR clones linked by an *Xba*I restriction site, which resulted in the replacement of the mucin sequence with two residues (serine-arginine). Mutations in isolated plasmid clones were identified by direct sequencing of mini-prep DNA using dye-terminator cycle sequencing reactions (ABI) analyzed on either an ABI 373 or 377 sequencer. Large-scale preparations for each type of mutated plasmid DNA were made using commercial kits (Promega Corp. or 5 Prime → 3 Prime, Inc.). The DNA was quantified by UV_{260} absorbance readings, and then stored at -70°C until needed. The coding region (*Bam*HI/*Sal*I fragments) from the plasmid pTM1(ΔNcoI)-GP and mutated versions of this DNA were separately ligated into the *Bam*HI/*Xho*I polylinker sites of the vector pcDNA3 (Invitrogen),

cloned in *E. coli*, and plasmid DNA isolated for use in pseudotyping studies.

VV-T7 expression of GP sequences. The plasmid pTM1(Δ*Nco*I)-GP, mutated versions of this clone, and the pTM1(Δ*Nco*I) vector (negative control) were introduced into 293 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase (5). Cells were cultured in 12-well panels to 80% confluence, then infected with vTF7-3 for 1.5 hr at a multiplicity of infection ≥10 using a purified virus preparation diluted in growth medium. Plasmid DNA was then introduced into infected cells by transfection. Transfection was performed by incubating a mixture of 300 µl DMEM (minus antibiotics or serum), 1.5 µg plasmid DNA, and 9.0 µl of Transfast (Promega Corp.) for 15 min at room temperature, then adding the mixtures to naked monolayers of vTF7-3-infected 293 cells that had been gently washed twice with DMEM. Cells were cultured for 1 hr, and then 1 ml growth medium added. Cells were cultured for an additional 5 hrs, and the medium replaced with 250 µl of Eagles minimal essential medium minus cysteine (plus antibiotics and 2% dialyzed fetal bovine serum) containing 150 µCi/ml [^{35}S]cysteine. After 3 hrs of culturing, 300 µl of growth medium was added to each well and culturing was continued for 14 hrs. At that time, supernatant fluids were removed and mixed with 66 µl 10X TNE buffer (0.1M Tris-HCl pH 7.4, 1.5 M NaCl, 0.02 M EDTA) containing 10% Triton X-100 (TX-100) and 10 mM phenylmethylsulfonyl fluoride (PMSF). Cell monolayers were lysed by adding 1 ml of 1X TNE containing 1% TX-100 and 1 mM PMSF to each well and incubating at room temperature for 5 min. After transfer to 1.5 ml Eppendorf tubes, the lysates were subjected to brief centrifugation in a microfuge (10,000 rpm) to pellet the nuclei. The supernatant fluids were then transferred to new tubes. GP molecules were immunoprecipitated from culture supernatant fluids and cell lysates by the addition of 100 µl of a 10% Staphylococcal protein A bacterial absorbent (Boerhinger-Mannheim) that had been preincubated for 15 min (with constant

mixing) with a rabbit-anti-Ebola SGP/GP serum (20); the IgG from 4.0 µl of serum was bound to each 100 µl volume of bacterial absorbent. Reactions were incubated at room temperature with constant mixing for 1 hr, then bacterial cells were washed by three rounds of centrifugation (10,000 rpm) and suspension in 1 ml 1X TNE containing 0.5% sodium deoxycholate and 0.5% Nonidet P-40. Pelleted cells were suspended in 50 µl of 0.125 M Tris-HCl (pH 6.8) containing 2.5% sodium dodecyl sulfate (SDS), 12.5% sucrose, and 0.01% bromophenol blue. The cell suspension was then boiled for 2 min, pelleted for 1 min at 14,000 rpm in a microfuge, and equal volumes of supernatant fluids were transferred to duplicate sets of 1.5 ml Eppendorf tubes. One set of fluids was reduced by adding 2-mercaptoethanol to a concentration of 1% (v/v), whereas the other was left untreated (nonreduced). Equal amounts of protein radioimmunoprecipitated from the medium and from the cell monolayer were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels and visualized by autoradiography.

Retrovirus pseudotyping and virus transduction assays. Pseudotyped retrovirus particles consisting of the MuLV cores and the Ebola GP in their envelopes were produced by transfecting wild-type or mutated plasmid DNA into gpnlslacZ cells as previously described (25). Virus transduction of β-galactosidase activity into NIH 3T3 cells was determined as previously described (25). All data presented are the average of the results of at least three experiments.

Immunoblot analysis of Ebola virus glycoprotein expression, processing, and incorporation into pseudotyped retroviruses. Medium from transfected ϕNX cells (11, 18, 26) containing recombinant retroviruses were passed through a 0.45 µm filter and centrifuged through a 30% sucrose cushion at 25,000 RPM in a Beckman 50.2-Ti rotor in a Beckman SS-71 centrifuge. The fluid was aspirated from centrifuge tubes and discarded, and the virus pellet was suspended in 100µl of RIPA buffer (140 mM NaCl, 10 mM Tris HCl pH 8.0, 5 mM EDTA, 1%

Na deoxycholate, 1% Triton X-100, 0.1% SDS). Cells were treated with lysis buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100), and the cell lysates were then centrifuged in a microcentrifuge at 16,100 x *g* for ten minutes. The proteins in the cell lysate and the suspended viral pellet were each precipitated with a final concentration of 4% TCA for two minutes. The precipitated proteins were then centrifuged at 16,100 x *g* in a microcentrifuge for ten minutes. The supernantant fluid was aspirated and discarded. The pellet was suspended in an equal volume of 1 M Tris[hydroxymethy]-aminomethane and vortexed vigorously. Proteins whose glycosylation was analyzed were treated sequentially with PNGase F, which removes *N*-linked glycosylation, and with both Sialidase A and Endo-*O*-glycosidase (ProZyme , Inc.), which together remove *O*-linked glycosylation, following protocols provided by the supplier. The pellet suspension was then mixed with 1/6 the volume of 300 mM Tris pH 6.8, 60% glycerol (w/v), 4% SDS (w/v), 0.0012% bromophenol blue (w/v), 6% 2-mercaptoethanol (v/v) and boiled for 5 minutes. Equal amounts of proteins as determined by the Bradford assay were separated by SDS-PAGE (8.5% acrylamide), and electrophoretically blotted onto nitrocellulose membranes. Membrane blots were immersed in reaction buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) containing 1% bovine serum albumin and incubated overnight at 4°C. Blots were incubated in reaction buffer containing a rabbit-anti-Ebola SGP/GP diluted 1:1000 for 1 hr at room temperature, washed 3 times in reaction buffer, and reacted with a Goat anti-rabbit-horseradish peroxidase conjugate (diluted 1:20,000 in reaction buffer) for 30 min at room temperature. Membranes were washed as before, then treated with a commercial chemiluminescent substrate solution (Amersham Pharmacia Biotech), according to the protocols provided by the manufacturer. Specific reactivity to GP was visualized by exposing treated blots to X-ray film.

RESULTS

Biochemical studies of mutant Ebola virus glycoproteins. The roles of the covalent modifications of the Ebola virus glycoprotein were investigated through examination of the effects of mutations that would eliminate the modifications (Figure 1). The rationale for the experiments involving substitutions for the cysteine residues in the extracellular domains of the glycoprotein is based on the fact that cysteines in extracellular domains are conserved exclusively for thiol-disulfide chemistry (24). Pairs of conserved cystienes form disulfide bonds. Elimination of one of a pair of half-cystines should have the same structural effects as the elimination of the other. It follows that the substitution of one should produce similar effects on glycoprotein processing as the substitution of the other. Finally, elimination of both cysteines of a disulfide-bonded pair should have no additional effect upon glycoprotein processing or function than the elimination of either one alone. Indeed, the absence of a free cysteine in the doubly substituted glycoprotein might actually improve processing or function over that observed with glycoproteins that have been mutated so that they possess an unpaired cysteine and are thereby likely to be recognized and retained intracellularly by the secretory-pathway quality-control system (24).

The processing of mutant Ebola GPs was examined through radioimmunoprecipitation assays performed on GPs whose expression was induced by production of T7 RNA polymerase. by recombinant vaccinia virus (Fig. 2). In the cell lysate the wild-type protein is predominantly found in the form in which it is processed to GP_1 and GP_2, although some precursor protein that is not proteolytically processed (GP_{pre}) is detected. GP_1 molecules are shed into the medium at a level equal to that of cell-associated GP_1. Mutation of the first cysteine in GP_1 (cys-53) resulted in the secretion of most of GP_1 into the medium and a greater electrophoretic mobility for GP_2 (Fig. 2, 1st autoradiograph). Mutation of each of the remaining cysteines in GP_1 reduced levels of expression of GP_1 and GP_2, and the predominant form was the GP_{pre} molecule. Mutation of the second or fourth cysteines (cys-108 and cys-135) resulted in little or no GP_1 or GP_2 production, whereas plasmids with changes in the third or fifth cysteines (cys-121 and cys-147) generated small amounts of mature GP_1 and GP_2. Mutations in most of the conserved *N*-linked sites in GP_1

produced little change in expression levels and patterns (Fig. 2, 2nd autoradiograph). Elimination of the most amino-terminal site through substitution of an aspartate residue for asparagine-40 caused more GP_1 to be secreted into the medium than was associated with cells. These changes to the *N*-linked sites in GP_1 caused no apparent changes in the migration of GP_1 or GP_2.

Mutation of any of the first through the fifth cysteines of GP_2 led to markedly increased levels of GP_1 in the medium. GP_{pre} predominated in the cell lysate, and little or no normally processed GP_2 was produced (Fig. 2, 3rd autoradiograph). Only the C511G and C556S GP_2 molecules were easily detected, and they displayed a greater electrophoretic mobility that was similar in migration to the GP_2 resulting from the GP_1 C53G substitution. Substitution of the cys-672 in the GP_2 membrane-spanning region with phenylalanine resulted in only a slight diminution in expression of GP_1 and GP_2, whereas substitution of the nearby cys-670 residue or both cys-670 and cys-672 with phenylalanine produced more marked reductions in expression (Fig. 2, 4th autoradiograph).

The effect of mutation of each of the two conserved *N*-linked glycan sites of GP_2 depended on the site that was eliminated. When the first site was changed (asn-563), little GP_1, GP_2, or GP_{pre} was detected in the medium or the cell lysate (Fig. 2, 4th autoradiograph).

Mutation of the second site (asn-618) appeared to cause only a small reduction in expression, but the migration of GP_2 appeared significantly faster, presumably due to the loss in mass normally contributed by glycosylation at the site.

To determine if there was any disulfide bonding between GP_1 and GP_2 by GPs in which mutated cysteines led to increased GP_1 release into the culture medium, SDS-PAGE analysis of nonreduced GP preparations was performed (Fig. 3). The mobility of the mutant GPs indicated that no GP_1-GP_2 covalent heterodimers were formed. This indicates that these mutations affect disulfide bonding between GP_1 and GP_2, and that the most N-terminal cysteine residue GP_1 forms the cystine bridge with GP_2.

Pseudotyped retroviruses bearing GPs containing substitutions for conserved cysteines. In order to assay the functional consequences of the mutations in GP, we examined the incorporation of the mutant GPs into recombinant retroviruses and gene transduction by the pseudotyped viruses (Fig. 4 and Table 2). Mutation of cys-53, which we identified as being involved in GP_1-GP_2 cystine bridge formation, completely abolished incorporation of GP into pseudotyped retrovirus particles as well as transduction. Viruses bearing the C108G, C121G, C135S, or C147S GPs all conveyed lower levels of transduction than did the wild-type GP. All of these four mutant GPs exhibited a decrease in processing and incorporation into virus particles. We also examined the result of substituting two GP_1 cysteines simultaneously. Remarkably the virus bearing the C121G/C147S GP has nearly the same capacity to transduce cells as the wild-type GP (Table 2). This is despite the fact that the C147S GP could not confer transduction capacity on pseudotyped viruses. The C108G/C135S GP possessed very modest function.

Mutation of each of the ectodomain cysteines in GP_2 (cys-511, cys-556, cys-601, cys-608, and cys-609) resulted in a reduction in the ratio of cell-associated mature GP_1 to GP_{pre}, minimal incorporation of GP into retrovirus particles (Fig. 4), and the complete abolishment of transduction (Table 2). It is worth noting that similar levels of the processed forms of the C601S and C608S GPs were detected and that these amounts were greater than those of the processed forms of C511G, C556S and C609S GPs.

The two cysteines within the membrane-spanning sequence of GP_2 (C670 and C672) are palmitoylated (13). Substitution of either cys-670 or cys-672 or both with alanine residues did not have major effects upon GP processing and incorporation into retroviral particles (Fig. 5) nor did it affect function in the transduction assay (Table 2). The substitution of cys-670 with phenylalanine decreased transduction by 43%, and greatly reduced but did not eliminate GP processing and incorporation into pseudotyped particles. The C672F mutation led to a 24% decrease in transduction, and the glycoprotein was processed and incorporated into particles at near wild-type levels. The double mutant C670F/C672F was expressed and incorporated into viral particles at greatly diminished levels and had a complete loss of transduction capacity.

Pseudotyped retroviruses bearing GPs with altered glycosylation. The effects of mutating conserved *N*-linked glycosylation sites (N-X-T/S) on pseudotyping and transduction were measured (Fig. 6 and Table 3). It was found that processing and incorporation of the mutant GPs was similar to that of the wild-type GP at six of the eight sites mutated. However, mutation of asn-40 (first *N*-linked glycan), which is near the cysteine that forms the GP_1-GP_2 cystine bridge, and mutation of asn-296, which is on the cusp of the variable, mucin-like region, each resulted in a significant effect on viral transduction. The N40D mutation completely abrogated transduction (Table 3), and greatly reduced glycoprotein processing and incorporation into virus particles. In order to investigate further the role of glycan attachment at this site, a T42D mutation was engineered into the wild-type sequence, which would prevent glycan addition at asparagine-40. This mutation had no effect upon the transduction capacity of pseudotyped particles or upon the level of expression and electrophoretic mobility of the proteins. This suggests that the negative effects of the N40D mutation resulted not from the loss of glycosylation, but rather from conformational disruption produced by the substituted residue.

The role of *O*-glycosylation of the Ebola virus GP was examined through analysis of the effects of deleting the region of the protein that is *O*-glycosylated. Remarkably processing and viral incorporation of the Δ309-489 GP is greatly enhanced (Fig. 7), and there is a corresponding increase in transduction by the Δ309-489 GP pseudotyped viruses (Table 3). The absence of an increase in the mobility of the Δ309-489 GP upon sialadase A and endo-*O*-glycosidase treatment provides confirmation that the region of *O*-glycosylation of the glycoprotein has been removed (Fig. 8).

DISCUSSION

The combination of protein expression and recombinant virus studies has enabled us to study the roles of covalent modifications of the Ebola virus GP complex. The primary and quaternary structures of the Ebola virus glycoprotein have remarkable similarities to those of retrovirus glycoproteins (6, 10, 16, 36), and the results presented here support the hypothesis that additional remarkable common properties are shared.

The combination of sequence analysis, structure determination and the results presented in this article lead us to propose a model of the cystine bridge map of the Ebola GP (Fig. 9A). The extracellular domains of the Ebola virus glycoproteins contain 10 conserved cysteine residues, five in GP_1 and five in GP_2. The five cysteines in the Ebola virus GP_2 are conserved in not only the Marburg virus GP_2, but also in the avian sarcoma and leukosis virus (ASLV) TM glycoproteins, which are known to be linked by a stable disulfide bond to their SU components (Fig. 9B). On the basis of sequence analyses and X-ray diffraction studies of Ebola GP_2, a putative structure for the linkage of the Ebola virus GP_1 and GP_2 has taken shape. As we noted earlier, the first cysteine in GP_1 (cys-53) had been predicted to be linked to the last cysteine in the extracellular domain in GP_2 (cys-609) (10, 23). We have confirmed the involvement of the first cysteine in GP_1 in the disulfide linkage to GP_2. A substitution of a glycine for cys-53 led to the release of most of GP_1 into the medium in the VV-T7 expression system (Fig. 2), with no evidence of C53G GP_1 being disulfide-linked to GP_2 in either the medium or the cell (Fig. 3). This amino-terminal cysteine is conserved in the GP of filoviruses, and it is anticipated that it also links the Marburg virus GP_1 to GP_2.

Conformational changes resulting from the elimination of the GP_1-GP_2 cystine bridge probably produce the changes in processing that alter the mobility of GP_2 in polyacrylamide gels (Fig. 2). Substitutions for any of the GP_2 cysteines (cys-511, cys-556, cys-601, cys-608, and cys-609) all led to higher levels of secretion of the GP_1 in the VV-T7 experiments (Fig. 2), and inefficient processing and incorporation into recombinant Mo-MuLV (Fig. 4) particles and consequent abrogation of transduction capacity. We conclude from these data that when any of the

GP_2 cysteines are replaced with another residue, a disruption in the structure of GP_2 occurs that prevents the GP_1-GP_2 linkage from forming. These changes in GP_2 did not appear to affect the migration or level of production of GP_1 in the VV-T7 expression system (Fig. 2), suggesting that disulfide bonding between cys-53 and cys-609 is not absolutely required for the transport and processing of highly expressed GP_1, but increased GP_{pre} in the cell may point to some reduction in trafficking of GP to the Golgi. In the cell lysates of the retroviral pseudotype producer cells, on the other hand, processing of the five mutants was greatly reduced. The pronounced effects of the GP_2 C601S, C608S, and C609S substitutions on processing are similar to those obtained when the equivalent residues of the MuLV TM protein were altered (28).

It is noteworthy that in the pseudotype producer cells similar levels of the processed forms of the C601S and C608S GPs were detected and that these amounts were greater than those of the processed forms of the C511G, C556S, and C609S GPs (Fig. 4). Furthermore, in the VV-T7 expression experiments only the C511G and C556S GP_2 molecules were easily detected, and they displayed a similar altered electrophoretic mobility (Fig. 2). Given the premises of our experiments, these findings indicate that cys-601 and cys-608 are likely to form a cystine bridge, as predicted by the structural studies (16, 36), and that cys-511 and cys-556 form another (Fig. 9A). The filovirus GP_2 subunits and ASLV TMs contain an internal fusion peptide (residues 524 to 539 in the Ebola virus GP_2) that is flanked by cysteine residues that correspond to this latter pair (cys-511 and cys-556). These cysteines are absent from the TM glycoproteins of other retroviruses, which have the fusion peptide positioned very close to the furin cleavage site. The filovirus GP_2 and ASLV TM cystine bridge could help stabilize the stalk structure and fix the fusion peptide into a conformation that is favorable for membrane insertion. It should be noted that our results indicating processing defects for the C511G and C556S GPs in the pseudotype producer cells contrast somewhat with recently published data concerning the ASLV TM. It was demonstrated that substitution for residues in the ASLV TM that are equivalent to cys-511 and cys-556 does not affect ASLV glycoprotein processing (3). Nevertheless the entry of viruses bearing the altered glycoproteins was dramatically reduced. It appears likely that, although there are similar

structural consequences of elimination of the cystine bridge in the ASLV TM and Ebola GP, they manifest themselves earlier in the case of the Ebola glycoproteins.

It has been proposed, through analogy to the thiol-disulfide exchange reactions that take place in the MuLV glycoproteins (19, 24), that the disulfide bond described above between GP_1 and GP_2 may be reduced by a cellular enzyme upon entry of the Ebola virus (24). It is evident from our studies and those performed previously that a significant amount of GP_1 is normally released into the medium from cells infected with Ebola virus or expressing the GP gene transiently (33); dissociation of GP1 from GP2 could occur within the cell or when the peplomer is exposed on the cell surface. The apparent ease with which GP_1 is released from the peplomer could be advantageous to the virus, especially if the large globular GP_1 molecules interfere with later stages of virus entry.

Four cysteine residues in the Ebola GP_1 molecule, cys-108, cys-121, cys-135, and cys147, are likely to be linked in two intramolecular cystine bridges (Fig. 9A). The GP containing the C121G mutation showed the least impairment of function, which suggests that the disulfide bond partner of cys-121 may not be exposed on the surface (4, 24). It is noteworthy that the processing of the C121G and C147S GPs is similar in each of the two expression systems (Fig. 2 and Fig. 4), which, by the premises of our experiments would indicate that cys-121 and cys-147 form a disulfide bond in the wild-type protein. Further support for this prediction is provided by the finding that the C121G/C147S GP can convey transduction capacity on virus equivalent to that conveyed by the wild-type GP despite the low level of function of the C147S GP (Table 2). Consistent with this proposal is the probability of a cystine bridge between cys-108 and cys-135 of the Ebola GP_1. These residues are also conserved in the GP_1 molecules of all Marburg virus isolates. Together our data provide strong support for our proposed cystine-bridge map (Fig. 9A).

Virtually all of the *N*-glycosylation sites in the Ebola glycoprotein that we eliminated were individually dispensable (Fig. 6, Table 3). These data are very similar to those obtained when the *N*-glycosylation sites of the MuLV env proteins were mutated (9, 14, 15). Our results bode well for structural studies in which reduction of protein glycosylation (and consequent heterogeneity) is

advantageous to protein crystallization. In this context it is worth noting the effects of eliminating the first site of GP_1 *N*-glycosylation, which precedes cys-53 by eleven residues. The mutant glycoprotein bearing the N40D substitution is secreted at higher levels in the VV-T7 expression system, is poorly processed and incorporated into the Mo-MuLV particles, and has no transduction capacity. However, it is not the *N*-glycosylation per se that is required, since a T42D substitution (which would also eliminate the first site of *N*-glycosylation) did not impair transduction.

There are conserved *N*-glycosylation sites in similar relative positions near the intersubunit half-cystines in the SU proteins of the MuLVs and homologous virus glycoproteins (14, 19), and it has been shown that an aspartate for asparagine substitution at this site leads to complete loss of function (9, 14). Remarkably, substitution of a valine for the threonine residue at this *N*-glycosylation site, which is similar to our GP_1T42D substitution, does not lead to such severe defects in function (15). It was concluded that although *N*-glycosylation at the conserved site was not required for infectivity, the conformation of this region of the polypeptide was critical for normal envelope protein processing and glycoprotein subunit association and that *N*-glycosylation played a role in these functions (15). It is likely that the conformational role of the first *N*-glycosylation site of Ebola GP_1 is similar. Although this site is conserved in all Ebola virus GP_1 molecules, an equivalent site in the GP_1 of Marburg virus is absent.

Significant deleterious effects on GP_1 and GP_2 processing were observed when the N563D substitution mutant was expressed in the VV-T7 system. The two GP_2 *N*-glycosylation sites may together play an important role in glycoprotein folding. Intra- and intermolecular disulfide-bond formation or reduction could be facilitated by the proximity of *N*-linked glycans to particular cysteine residues. In this context it is noteworthy that ER-localized thiol-disulfide exchange enzymes that promote disulfide-bond formation in secreted proteins appear to be found in a complex with the *N*-glycan-binding ER chaperones calnexin and calreticulin (17). It is interesting that the Rous sarcoma virus TM also has *N*-glycosylation sites near the ectodomain cysteine residues (Fig. 9B) and that some of the retroviral TMs that have their fusion peptides positioned at the extreme amino-terminus have only one predicted *N*-glycosylation site, which is located near the

cysteine involved in forming the SU-TM linkage.

The effect of deleting the *O*-glycosylation region of GP_1 (Δ309-489) on expression and transduction were striking. This segment, which is rich in proline, serine, and threonine residues is the most variable among the Ebola GPs. Elimination of this mucin-like domain results in enhanced GP processing and incorporation into retroviral particles (Fig. 7) and consequently higher levels of transduction by the pseudotyped retroviruses (Table 3). It is possible that the wild-type GP is retained in the Golgi apparatus until all of the serines and threonine residues in the mucin region are modified. Elimination of this segment may permit more rapid transit through the Golgi apparatus and higher levels of processing to GP_1 and GP_2 and of cell-surface expression. Increased viral incorporation may also result from a diminution of GP toxicity. It has been reported that the deletion of the *O*-glycosylation region reduces the cytopathic effects of Ebola virus GP expression (40). It has also been suggested that the expression of high levels of the wild-type Ebola GP might lead to exhaustion of the cellular glycosylation machinery (32), which is consistent with our results and our interpretation. In any case, the improved levels of transduction by the virus pseudotyped with the Δ309-489 GP, combined with its potential safety advantages, should make such recombinant virus the first choice for gene therapies utilizing Ebola GP-pseudotyped retroviruses or lentiviruses.

The conservation of a mucin domain and its variability between isolates indicates that it is likely to be playing a critical in the ecology and pathogenesis of the virus, which cannot be assessed in the pseudotype system. The probable surface exposure of the charged sugar moieties in this region might make it a dominant target for the humoral immune system. The fact that it is apparently not participating in viral entry could mean that antibodies against it are not neutralizing. In addition, its toleration for variation could allow the virus to readily escape immune recognition through mutation. The mucin-like qualities of the outer surface of the peplomer, along with its size, may also act as a barrier to immune recognition and virus clearance.

The presence of cysteines in membrane-spanning regions appears to be a conserved feature of many virus glycoproteins, including the TMs of retroviruses. Analysis of the effects of

substitutions of the conserved cysteine residues of the membrane-spanning sequence of Ebola virus GP_2, indicates that the nature of the substituted residue is critical. Substitutions of alanine residues for either cys-670 or cys-672 or both led to no major consequences in our assays. These results are similar to those obtained in a system where vesicular stomatitis virus was pseudotyped with mutant Ebola GPs (13). Substitutions of phenylalanine for either cys-670 or cys-672 had only moderate effects on transduction titers, whereas mutant Ebola glycoproteins bearing substitutions in both sites were poorly expressed and incapable of promoting transduction (Table 2).

Our biochemical data confirm the significance of the sequence similarity between the filovirus GP_2 and the TM of oncogenic retroviruses, in particular, the TM of ASLVs (10). These findings support the hypothesis of a common evolutionary origin. The fact that the retrovirus TMs that show the greatest similarity to filovirus GP_2 molecules come from birds could be an indication that avian species have or have had some role in the ecology and evolution of filoviruses.

In conclusion, this study has provided the basis for a greater understanding of the structure and function of the Ebola GP, and has enhanced our appreciation of the relationship between the filovirus and retrovirus glycoproteins. We have demonstrated the importance of the conserved cysteines of the Ebola virus GP on its processing, assembly of the peplomer, and virus entry. Although individual conserved *N*-glycosylation sites were not found to be as important as the conserved cysteines, it is expected that collectively they have a strong influence on protein folding and disulfide bond formation. Our finding that the elimination of the region of *O*-glycosylation of the Ebola GP enhances its processing, incorporation into retrovirus particles and transduction by pseduotyped retroviruses could have major implications for gene-therapy applications. We anticipate that further analyses of the Ebola virus GP will provide additional insights into the mechanisms of virus entry for filoviruses and other pathogenic viruses.

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Figure Legends

FIG. 1 Schematic representation of the Ebola virus glycoprotein. The GP_1 and GP_2 subunits of the glycoprotein are drawn to scale (residue numbers are indicated below the diagram). Positions of the signal sequence (diagonal bars), conserved cysteine residues (S), the mucin-like region (region of *O*-linked glycosylation, black), furin cleavage site, the fusion peptide (vertical bars), the coiled-coil domain (diagonal bars), and the membrane-spanning domain (horizontal bars) are indicated.

FIG. 2. Radioimmunoprecipitation of GP in 293 cells. The GP proteins encoded by the plasmids listed in Table 1 were expressed in 293 cells using a VV-T7 system and radiolabeled with [^{35}S]cysteine. They were then immunoprecipitated, and the reduced proteins were analyzed on 10% SDS-PAGE gels under reducing conditions, and autoradiography was performed. Immunoprecipitated GP secreted/released into the medium (M) or associated with the cell monolayer (L) were run side-by-side; only the relevant portions of the gels are shown. The migration positions of GP_1, GP_2, and GP_{pre} are identified in the left margin; GP_{pre} is an uncleaved immature or precursor form of GP that is primarily associated with the ER (27). Asterisks next to GP_1 identify increased levels of this glycoprotein in the medium relative to cell-associated GP_1, as compared to the wild type. Asterisks in the GP_2 region identify faster migrating forms of GP_2. There are cross-reactive species migrating just slower and somewhat faster than the wild-type GP_2.

FIG. 3. Migration of GP under nonreducing conditions. An autoradiogram of the SDS-PAGE analysis (under nonreducing condtions) of the wild-type GP and of those proteins analyzed in Figure 2 that demonstrated increased release of GP_1 into the medium. Immunoprecipitated GP secreted/released into the medium (M) or associated with the cell monolayer (L) were run side-by-side. The migration positions of GP_1, GP_2, and GP_{pre} are identified in the left margin

FIG. 4. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola GPs with substitutions of ectodomain cysteine residues. ϕNX cells were transfected with plasmids encoding Ebola GPs. The cell lysates (L) and viral particles collected from the culture medium (M) were analyzed by SDS-PAGE (8.5% acrylamide) and immunoblotting utilizing anti-Ebola SGP/GP antibody. Analysis of an aliquot of the cell lysates that was treated with PNGase F (+), which removes *N*-linked glycosylation, is also presented. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform) , GP_{pre} (the *N*-glycosylated but not *O*-glycosylated uncleaved glycoform) and deglycosylated GP_0 and GP_{pre} is indicated.

FIG. 5. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola GPs with substitutions of membrane-spanning-domain cysteine residues. Analysis was conducted as described in Figure 4. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform) , GP_{pre} (the *N*-glycosylated but not *O*-glycosylated uncleaved glycoform) and deglycosylated GP_0 and GP_{pre} is indicated.

FIG. 6. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola GPs with substitutions eliminating sites of *N*-glycosylation. Analysis was conducted as described in Figure 4 except that no PNGase F was performed. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform) , GP_{pre} (the *N*-glycosylated but not *O*-glycosylated uncleaved glycoform) and GP_2 is indicated.

FIG. 7. Analysis of the expression and incorporation into pseudotyped retroviruses of the Δ309-489 Ebola GP . Analysis was conducted as described in Figure 4. The migration of the mature GP_1, GP_0 (the glycosylated but uncleaved glycoform) , GP_{pre} (the *N*-glycosylated but not *O*-glycosylated uncleaved glycoform) and deglycosylated GP_0 and GP_{pre} forms of the wild-type GP and of the GP_1, GP_0 and deglycosylated GP_1 and GP_0 forms of the Δ309-489 GP is indicated.

FIG. 8. Analysis of the glycosylation of the Δ309-489 Ebola GP incorporated into pseudotyped retroviruses. Analysis was conducted as described in Figure 4 except that aliquots of the samples were also treated with PNGase F and endo-*O*-glycosidase or a combination of PNGase F, endo-*O*-glycosidase, β(1-4) galactosidase and glucosiminidase. The migration of the mature GP_1 of the wild-type and Δ309-489 GPs is indicated. In this experiment a glycosylated serum protein possessing a mobility intermediate between those of the wild-type and Δ309-489 GP_1s is detected. The heterogeneous mobility of the PNGaseF-treated proteins is indicative of incomplete removal of *N*-glycosylation.

FIG. 9. Cystine-bridge model of the Ebola virus GP and comparison of GP_2 to the Rous Sarcoma Virus glycoprotein TM subunit. Representational elements for signal sequence, the mucin-like region, the fusion peptides, the coiled-coil domains and the membrane-spanning domains are identical to those used in Figure 1. (A) The cystine bridge arrangement in the Ebola virus GP deduced from the results presented here and the critical *N*-glycosylation sites (Y) discussed in the text are depicted. (B) The proposed cystine-bridge model for the TM protein of Rous sarcoma virus (an ASLV) is presented for comparison with that of the Ebola virus GP_2.

TABLE 1. Mutant Ebola GPs expressed using the VV-T7 system

C53G (GP_1cys #1)	C511G (GP_2cys #1)
C108G (GP_1cys # 2)	C556S (GP_2cys#2)
C121G (GP_1cys #3)	C601S (GP_2cys #3)
C135S (GP_1cys #4)	C608S (GP_2cys #4)
C147S (GP_1cys #5)	C609S (GP_2cys #5)
N40D (GP_1 ΔN-glycan #1)	C670F (GP_2cys #6)
N204D (GP_1ΔN-glycan #2)	C672F (GP_2cys #7)
N238Y (GP_1ΔN-glycan #4)	C670F/C672F (GP_2cys #6+#7)
N257D (GP_1ΔN-glycan #5)	N563D (GP_2ΔN-glycan #1)
N277D (GP_1ΔN-glycan #6)	N618D (GP_2ΔN-glycan #2)
N296D (GP1 ΔN-glycan #7)	

TABLE 2: Transduction of NIH 3T3 cells by virus pseudotyped with mutant Ebola GPs with substitutions for cysteine residues

Mutant Glycoprotein	Transduction (% wild-type[a])
C53S	<0.1%
C108G	8.2 +/- 4.3%
C121G	59 +/- 8%
C135S	<0.1%
C147S	2.3 +/- 2.0%
C108G/C135S	1.1 +/- 1.0%
C108G/C147S	<0.1%
C121G/C135S	0.5 +/- 0.5%
C121G/C147S	72 +/- 30%
C511G	<0.1%
C556S	<0.1%
C601S	<0.1%
C608S	<0.1%
C609S	<0.1%
C670F	57 +/-7%
C672F	76 +/- 4.0%
C670F/C672F	<0.1%
C670A	113 +/- 21%
C672A	98 +/- 28%
C670A/C672A	82 +/- 15%

[a]Average transduction by virus bearing wild-type GP was 1.5×10^4 TU/ml

TABLE 3. Transduction of NIH 3T3 cells by virus pseudotyped with mutant Ebola GPs with altered glycosylation

Mutant Glycoprotein	Transduction (% wild-type[a])
N40D	<0.1%
T42D	113 +/- 17%
N204D	102 +/- 14%
N238Y	88 +/- 4%
N257D	88 +/- 9%
N277D	84 +/- 10%
N296D	62 +/- 10%
N563D	80% +/- 4%
N618D	102 +/- 3%
Δ309-489	696 +/- 142%

[a]Average transduction by virus bearing wild-type GP was 1.4×10^4 TU/ml

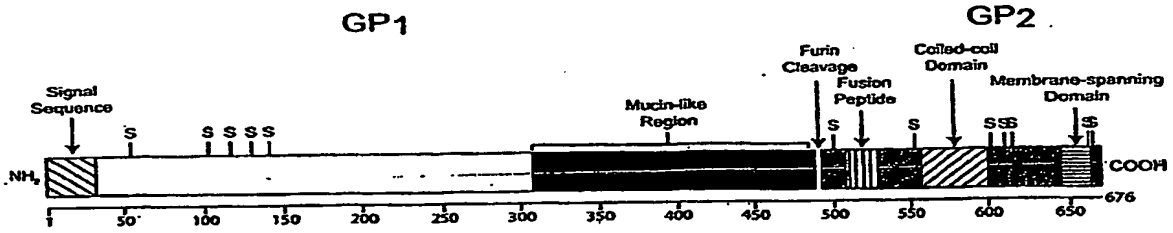

GP1
GP2
Signal Sequence
Mucin-like Region
Furin Cleavage
Fusion Peptide
Coiled-coil Domain
Membrane-spanning Domain
S
NH2
COOH
1
50
100
150
200
250
300
350
400
450
500
550
600
650
676

Fig. 1

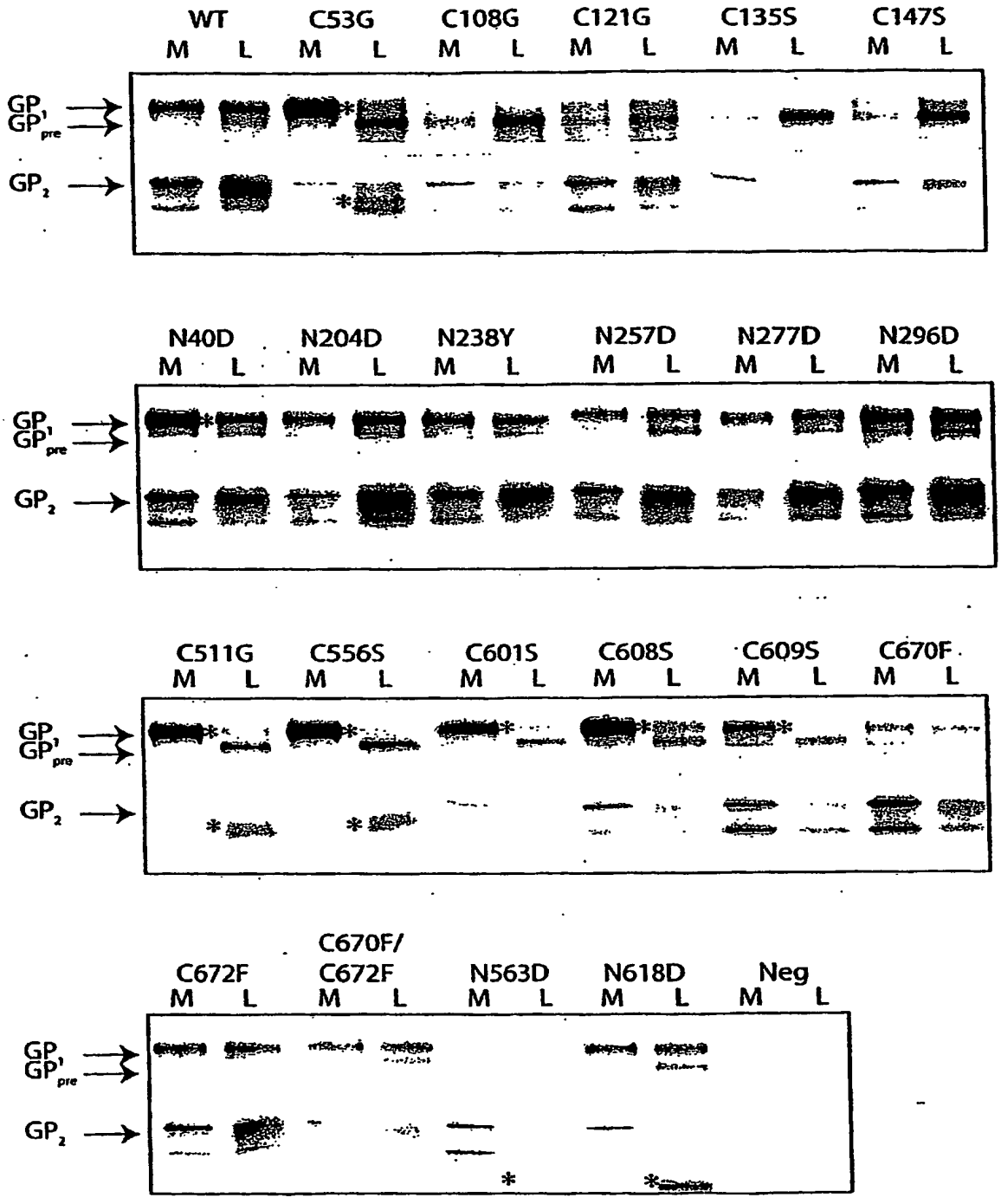

WT
C53G
C108G
C121G
C135S
C147S
M L M L M L M L M L M L
GP'
GP'pre
GP2
N40D
N204D
N238Y
N257D
N277D
N296D
M L M L M L M L M L M L
GP'
GP'pre
GP2
C511G
C556S
C601S
C608S
C609S
C670F
M L M L M L M L M L M L
GP'
GP'pre
GP2
C672F
C670F/
C672F
N563D
N618D
Neg
M L M L M L M L M L
GP'
GP'pre
GP2

Fig. 2

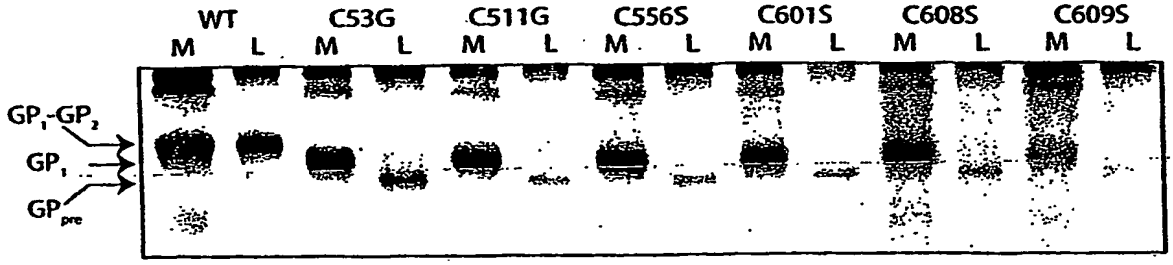
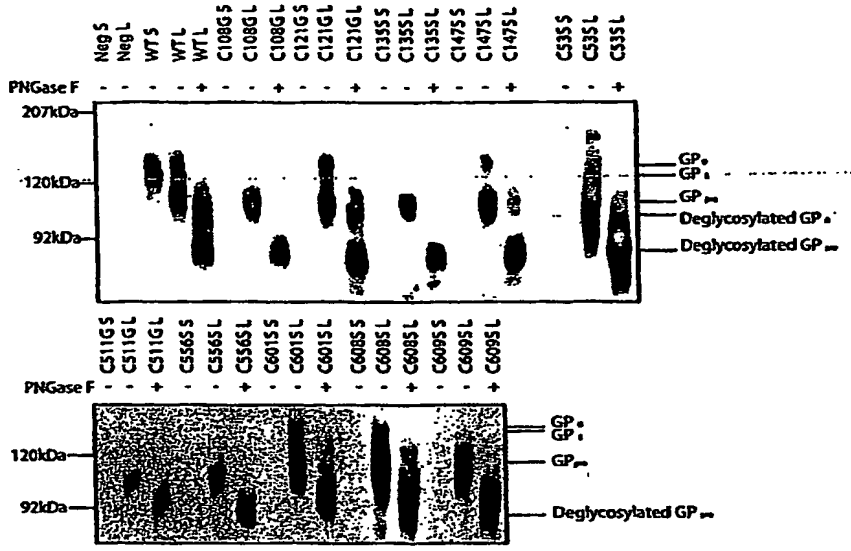


Fig. 3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Neg L
WT S
WT L
WT L
C108G S
C108G L
C108G L
C121G S
C121G L
C121G L
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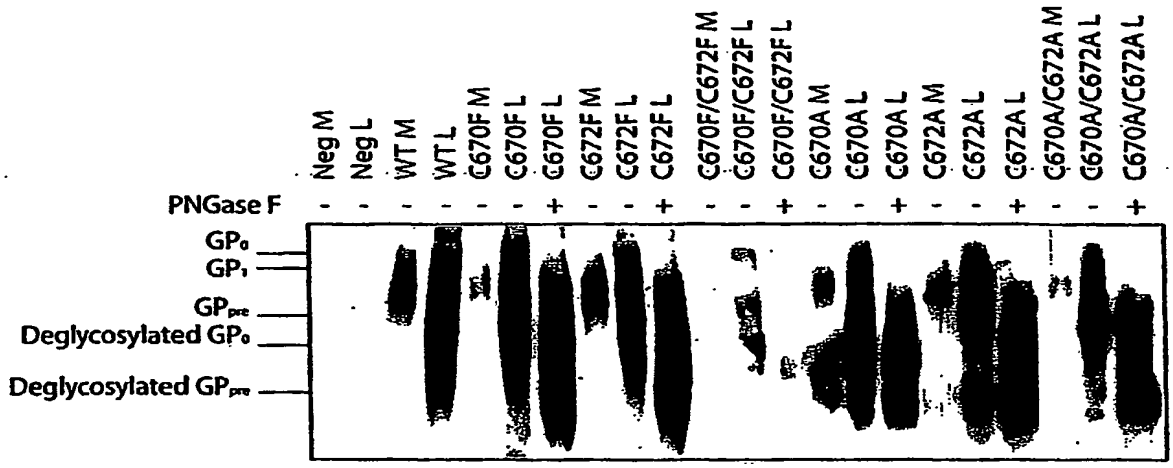
C133S L
C147S S
C147S L
C147S L
C53S S
C53S L
C53S L
PNGase F
207kDa
120kDa
92kDa
GP
GP
GP
Deglycosylated GP
Deglycosylated GP
C511G S
C511G L
C511G L
C556S S
C556S L
C556S L
C601S S
C601S L
C601S L
C608S S
C608S L
C608S L
C609S S
C609S L
C609S L
PNGase F
120kDa
92kDa
GP
GP
GP
Deglycosylated GP

Fig. 4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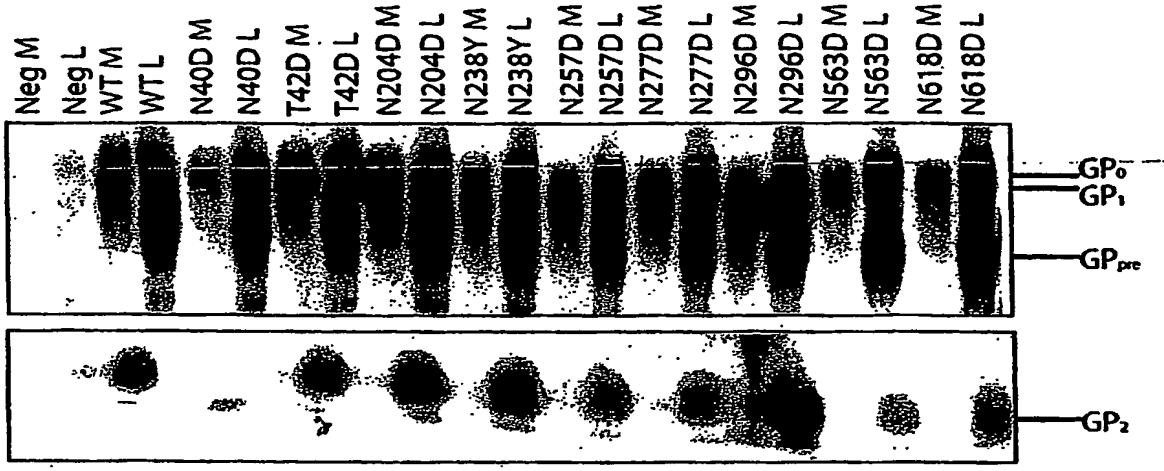
Neg L
WT M
WT L
C670F M
C670F L
C670F L
C672F M
C672F L
C672F L
C670F/C672F M
C670F/C672F L
C670F/C672F L
C670A M
C670A L
C670A L
C672A M
C672A L
C672A L
C670A/C672A M
C670A/C672A L
C670A/C672A L
PNGase F
- - - - - - + - - + - - + - - + - - + - - +
GP0
GP1
GPpre
Deglycosylated GP0
Deglycosylated GPpre

Fig. 5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Neg L
WT M
WT L
N40D M
N40D L
T42D M
T42D L
N204D M
N204D L
N238Y M
N238Y L
N257D M
N257D L
N277D M
N277D L
N296D M
N296D L
N563D M
N563D L
N618D M
N618D L
GP0
GP1
GPpre
GP2

Fig. 6

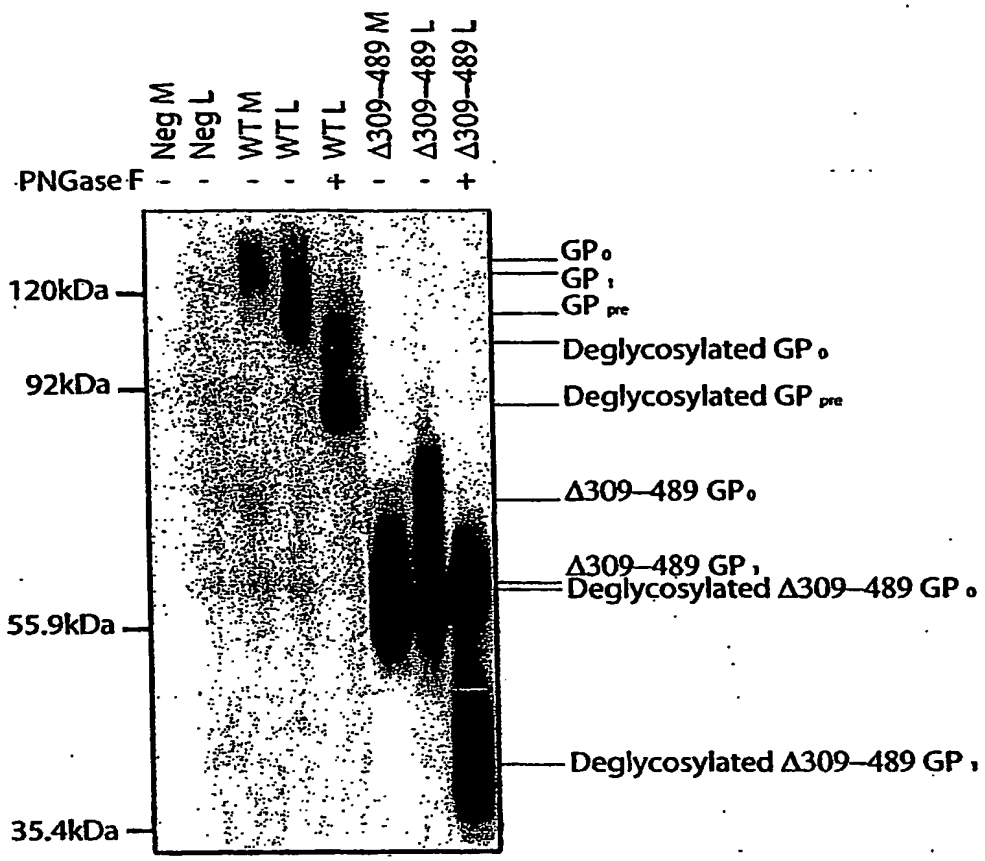

Neg M
Neg L
WT M
WT L
WT L
Δ309–489 M
Δ309–489 L
Δ309–489 L
PNGase F - - - - + - - +
120kDa
92kDa
55.9kDa
35.4kDa
GP0
GP1
GPpre
Deglycosylated GP0
Deglycosylated GPpre
Δ309–489 GP0
Δ309–489 GP1
Deglycosylated Δ309–489 GP0
Deglycosylated Δ309–489 GP1

Fig. 7

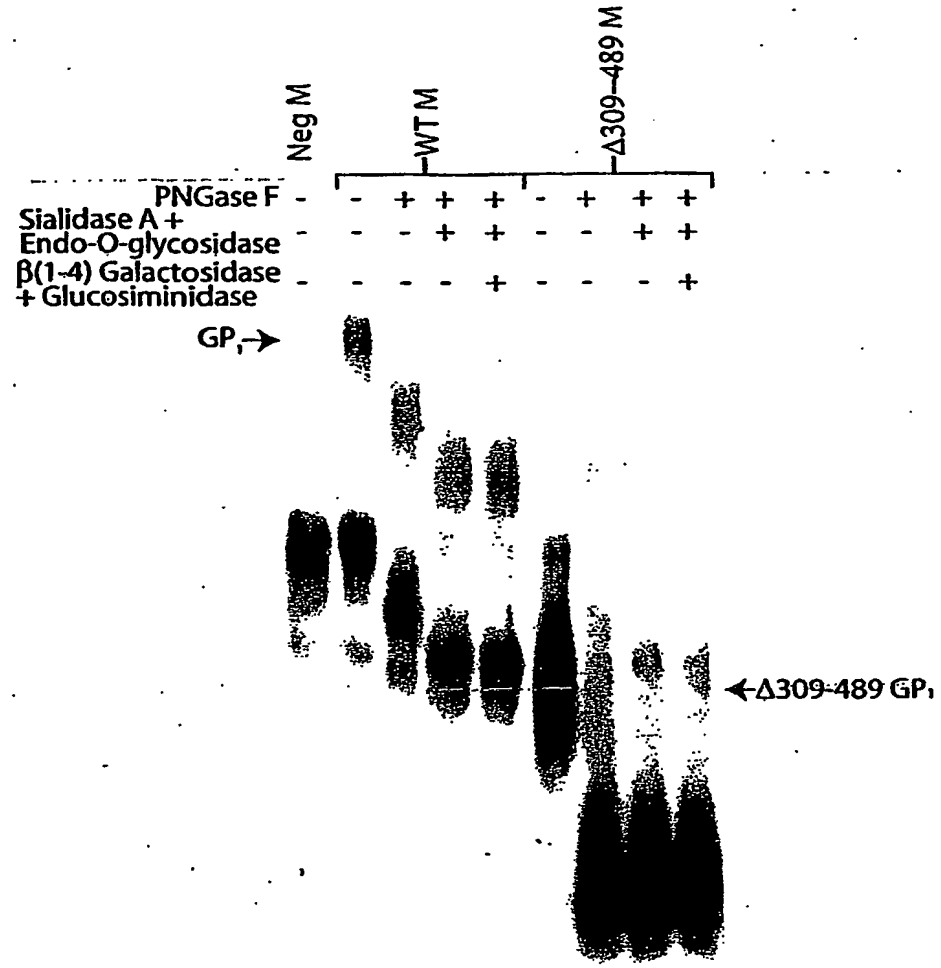
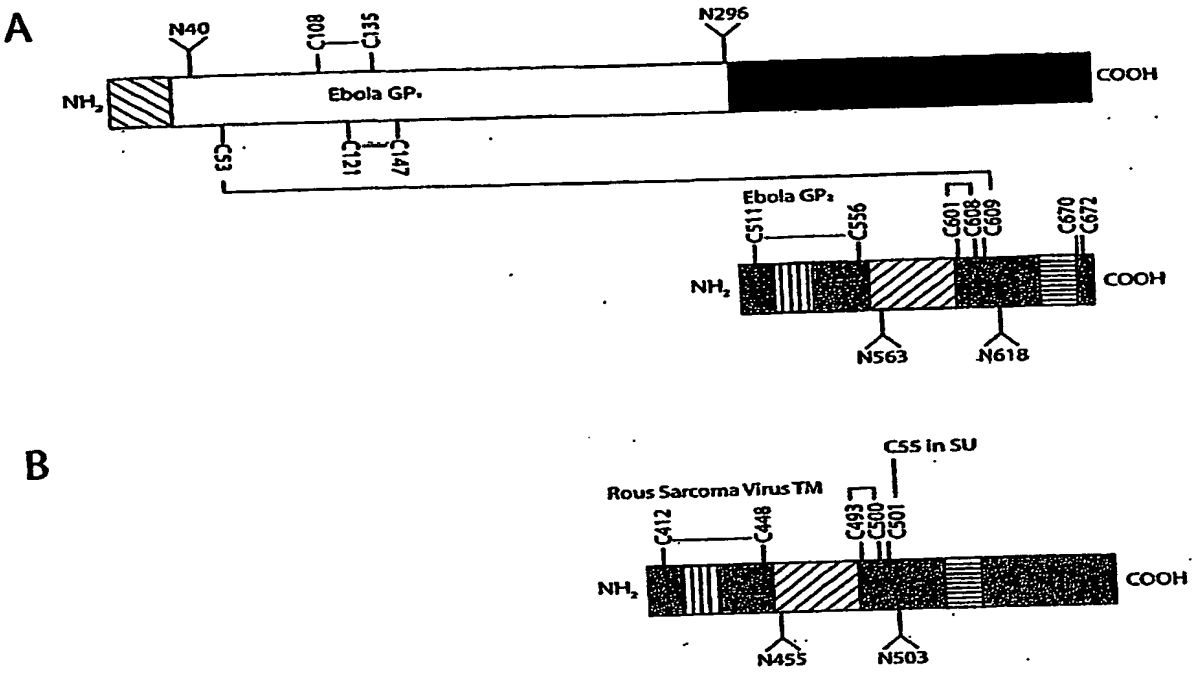

Neg M
WT M
Δ309–489 M
PNGase F - - + + + - + + +
Sialidase A + - - - + + - - + +
Endo-O-glycosidase
β(1-4) Galactosidase - - - - + - - - +
+ Glucosiminidase
GP1
Δ309-489 GP1

Fig. 8


A
N40
C108
C135
N296
NH2
Ebola GP1
COOH
C53
C121
C147
Ebola GP2
C511
C556
C601
C608
C609
C670
C672
NH2
COOH
N563
N618

B
C55 in SU
Rous Sarcoma Virus TM
C412
C448
C493
C500
C501
NH2
COOH
N455
N503

Fig. 9

The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for example, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In addition to the documents cited above, the following documents are also incorporated by reference: WO 00/08131 (Sanders et al., published February 17, 2000) and Sharkey et al., J. Virol. 75:2653-2659 (March, 2001).

What is claimed is:

1. A pseudotyped retrovirus comprising one or more elements as described herein.

2. A cell for producing a pseudotyped retrovirus, the cell comprising one or more elements as described herein.

METHODS FOR GENE TRANSFER USING PSEUDOTYPED LENTIVIRUSES

FIELD OF THE INVENTION

This invention relates generally to methods for gene transfer to cells using pseudotyped lentiviruses and more specifically to methods for gene transfer using togaviral and filoviral glycoprotein-pseudotyped lentiviruses.

BACKGROUND OF THE INVENTION

Gene therapy is one of the fastest growing areas in experimental medicine. However most studies are only Phase I or Phase II clinical studies designed mainly to evaluate the toxicity of the viral vectors and constructs being used. A major drawback has been the design of vectors that are both safe and efficacious.

Recently retroviruses have generated a great deal of interest for use as viral vectors. One major drawback for retroviral vectors designed to date is their inability to transduce non-dividing cells, such as airway epithelium, hepatocytes and brain glial cells. Retroviral vectors used in *ex vivo* and *in vivo* transduction of hepatocytes required inducing the hepatocytes to proliferate by complex and artificial procedures. One clinical trial was conducted to treat familial hypercholesterolemia by retroviral-mediated *ex vivo* gene transfer. The LDL receptor gene was introduced into hepatocytes that had been surgically removed from patients and which were then reinfused into the liver following gene transduction. There was no convincing evidence, however, of therapeutic efficacy. Liver biopsies were removed after treatment, and few cells tested positive for the expression of LDL-receptor, indicating that transduction efficiency was not high. *In vivo* retroviral-mediated transduction of hepatocytes was even more complicated, as it required artificial regeneration of the liver to give dividing cells. Ferry, N. et al., *Hum. Gene Ther.* 9:1975 (1998).

Retroviral vectors offer several potential advantages for attaining persistent expression of a therapeutic gene in airway epithelium for diseases such as cystic fibrosis. However, several problems have limited their application.

The airway epithelium possesses several unique properties that make it a formidable target for successful gene transfer. Among these are the many innate and adaptive host defense functions that the epithelium and resident immune effector cells perform. The pulmonary epithelium has evolved to prevent the invasion of the host

by microbes and these same strategies may act as barriers for gene transfer vectors. Advances in the field of gene transfer to airway epithelial cells have occurred as an understanding of the cell biology of epithelial host defenses and virus-cell interactions has increased.

Recombinant vectors based on Moloney murine leukemia virus (MoMLV) were the first retroviruses used for gene transfer to airway epithelium. Several studies demonstrated the potential for MoMLV-based vectors to persistently transduce airway epithelium by showing that the retroviral vectors could transduce airway epithelium *ex vivo* and furthermore, that the Cl-transport defect in cystic fibrosis (CF) airway epithelial cells was corrected by transducing the cells *in vitro* with a MoMLV retrovirus vector expressing the CFTR cDNA. However, MoMLV-based vectors require cell division in order for the integration complex to enter the nucleus. However, the normal airway epithelium is mitotically quiescent with less than 1% of the cells dividing. Therefore, transduction efficiency is low in airway epithelial cells.

Thus it would be desirable to have a retroviral vector that can efficiently transduce non-dividing cells, particularly hepatocytes, brain glial cells airway epithelial cells. It would be further desirable if such vectors were efficient in transducing hepatocytes, brain glial, and airway epithelial cells *in vivo*.

SUMMARY OF THE INVENTION

Provided in the present invention are methods for introducing nucleic acid sequences encoding a desired protein into a hepatocyte, brain glial or airway epithelial cell using a filoviral or togaviral glycoprotein pseudotyped lentiviruses. In one embodiment, the viruses can be used *in vitro* to introduce a nucleic acid sequence into a cell. In another embodiment, the viruses of the present invention are used for *in vivo* introduction of a nucleic acid sequence into a hepatocyte, brain glial or airway epithelial cell. In yet another embodiment, the nucleic acid sequence encodes CFTR and the cells are airway epithelial cells and the methods further comprise application of the pseudotyped virus to the apical surface of the airway. In an alternate embodiment the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. The methods further comprise application of the pseudotyped lentivirus to the liver and the brain.

Filoviral and togaviral glycoprotein-pseudotyped lentiviruses are provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein or two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment the filoviral glycoprotein is a Marburg or Ebola virus glycoprotein. In a further embodiment the Marburg virus glycoprotein has a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. The Marburg virus glycoprotein can have a C671A or a Y679 stop mutation. Pseudotyped virus comprising Marburg virus glycoprotein with at least one of these mutations have at least a two-fold increase in virus titer production. In an alternate embodiment, the togaviral glycoproteins are alphavirus glycoproteins, preferably the E1 and E2 envelope glycoproteins of Ross River virus (RRV).

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and by referencing the following drawings in which:

Figure 1A is a photograph of the en face view of duplicate samples of x-gal stained liver slices showing the efficiency of transducing hepatocytes with RRV pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1B is a photograph of the en face view of duplicate samples of x-gal stained liver slices showing the efficiency of transducing hepatocytes with VSV-G pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1C is a photograph of the en face view of duplicate control samples of x-gal stained liver slices treated with PBS;

Figure 1D is a photograph of a liver slice stained with hematoxylin and eosin showing efficiency of transducing hepatocytes with RRV pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1E is a photograph of a liver slice stained with hematoxylin and eosin showing efficiency of transducing hepatocytes with VSV-G pseudotyped FIV having a β-galactosidase reporter gene;

Figure 1F is a photograph of a control liver slice stained with hematoxylin and eosin which was treated with PBS;

Figure 2A is a bar graph showing the effect of RRV pseudotyped FIV and VSV-G pseudotyped FIV on liver function as measured by serum SGPT levels;

Figure 2B is a bar graph showing the effect of RRV pseudotyped FIV and VSV-G pseudotyped FIV on liver function as measured by serum SGOT levels;

Figure 3A is a photograph of showing the production of β-galactosidase by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 3B is a photograph of showing the production of GFAP by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 3C is a photograph of showing the production of β-galactosidase and GFAP by astrocytes transduced with RRV-pseudotyped FIV ;

Figure 4A is a photograph showing the production of β-galactosidase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 4B is a photograph showing the production of CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 4C is a photograph showing the production of β-galactosidase and CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5A is a photograph showing the production of β-galactosidase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5B is a photograph showing the production of CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 5C is a photograph showing the production of β-galactosidase and CNPase by oligodendrocytes transduced with RRV-pseudotyped FIV;

Figure 6 is a table showing the selective transduction of CNS cell types by FIV vectors pseudotyped with RRV envelope glycoproteins.

Figure 7A is a photograph showing gene transfer in human airway epithelia that were exposed on the apical surface to FIV pseudotyped with VSV-G;

Figure 7B is a photograph showing gene transfer in human airway epithelia that were exposed on the basolateral surface to FIV pseudotyped with VSV-G;

Figure 7C is a photograph showing gene transfer in human airway epithelia that were exposed on the apical surface to FIV pseudotyped with Marburg glycoprotein;

Figure 7D is a photograph showing gene transfer in human airway epithelia that were exposed on the basolateral surface to FIV pseudotyped with Marburg glycoprotein;

Figure 8 is a schematic showing mutations at the C-terminus of the amino acid sequences of the Marburg envelope glycoprotein (SEQ. ID. NOs: 1-7);

Figure 9 is a bar graph showing the effect of mutations at the C-terminus of the Marburg envelope glycoprotein on the titer of FIV pseudotyped with the mutant Marburg glycoproteins; and

Figure 10 is a schematic showing the amino acid sequence (SEQ. ID. NO: 8) of the Marburg envelope glycoprotein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for introducing nucleic acid sequences encoding a desired protein into a hepatocyte, brain glial or airway epithelial cell using a filoviral or togaviral glycoprotein pseudotyped lentivirus. In one embodiment, the viruses can be used *in vitro* to introduce a nucleic acid sequence into a cell. In another embodiment, the viruses of the present invention are used for *in vivo* introduction of a nucleic acid sequence into a hepatocyte, brain glial or airway epithelial cell. In yet another embodiment, the nucleic acid sequence encodes CFTR and the cells are airway epithelial cells and the methods further comprise application of the pseudotyped virus to the apical surface of the airway. In an alternate embodiment the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. The methods further comprise application of the pseudotyped lentivirus to the liver and the brain.

Filoviral glycoprotein-pseudotyped lentiviruses are provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein. In

one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment the viral glycoprotein is a Marburg virus glycoprotein. The Marburg virus glycoprotein can have a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. In a further embodiment, the Marburg virus glycoprotein has a C671A or a Y679 stop mutation. Pseudotyped viruses comprising Marburg virus glycoprotein with at least one of these mutations have at least a two-fold increase in virus titer production. In yet another alternate embodiment, the glycoprotein is an Ebola virus glycoprotein.

It will be appreciated by those skilled in the art that conservative substitutions of amino acids can be made without substantially changing the activity or structure of a protein. In one embodiment, the cysteine at position 671 of the Marburg glycoprotein is replaced by an alanine, valine, glycine, isoleucine, or leucine (Figure 8, SEQ. ID.NO: 4). It has been shown that when the cysteine at position 671 is replaced by an aliphatic, non-polar amino acid, the titer of FIV pseudotyped with the mutant Marburg glycoprotein increases about at least 3-fold (Figure 9). In an alternate embodiment, the amino acid sequence of the Marburg glycoprotein is truncated at the C-terminus. In another embodiment, the amino acid sequence is truncated from about isoleucine 680 (I680Stop) to about phenylalanine 676 (F676Stop) (Figure 8, SEQ. ID. NO: 7)). Truncating the amino acid sequence of the C-terminus of the Marburg glycoprotein results in at least a 2-fold increase in the titer of FIV pseudotyped with the truncated glycoprotein (Figure 9).

Togaviral glycoprotein-pseudotyped lentiviruses are also provided for use in the methods of the present invention. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV) which has two togaviral glycoproteins imbedded into the lipid bilayer surrounding the capsid. Examples of, but not limited to, togaviral glycoproteins are alphavirus glycoproteins, preferably the E1 and E2 envelope glycoproteins of Ross River virus (RRV). It was recently reported that by manipulating the E1 and E2 RRV glycoproteins so that they were expressed by individual genes in a packaging cell system, a stable cell line producing an RRV-pseudotyped Moloney murine virus was obtained. Sharkey, C.M.. et al., *J. Virol.* 75:2653 (2001).

The pseudotyped viruses of the present invention may further comprise another nucleic acid sequence that encodes a desired protein. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleic acid sequence may be introduced into the pseudotyped virus in a variety of ways known to the skilled artisan. In one embodiment, the nucleic acid sequence encodes for CFTR (cystic fibrosis transmembrane regulator protein), the chloride transporter that is involved in cystic fibrosis. The absence of CFTR function in lung epithelium due to mutations in the gene encoding CFTR, results in a severe lung disease that cannot be readily reversed or controlled by conventional treatment. Lack of CFTR function in the lung results in airway fluid with an altered ion composition, thereby creating a favorable environment for disease-causing bacteria to colonize the lung. Additionally, mucus secreted into the lung becomes thick and viscous, preventing normal clearing of the bacteria from the airways. The chronic bacterial infection leads to destruction of lung tissue and loss of lung function. Replacing the defective gene with a copy that encodes for a functional CFTR can abate the symptoms. In an alternate embodiment, the nucleic acid sequence encodes for the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter. For example, increasing the expression of the LDL receptor in the liver allows for more efficient clearance of LDL-cholesterol from the body.

Alternatively, the desired protein may be one that allows the entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleic acid sequences include the nucleic acid sequence encoding the *Aequorea victoria* green fluorescent protein and the LacZ gene that encodes for beta-galactosidase, both of which are well known in the art and may be obtained commercially.

Methods of introducing a nucleic acid sequence encoding a desired protein into a cell are provided. In one embodiment, the method includes contacting, or transducing, an airway epithelial cell with a lentivirus that has been pseudotyped with

a filoviral glycoprotein that includes the desired nucleic acid sequence in its genome. The level of transduction may be monitored by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleic acid sequences or assaying for the presence of the nucleic acid sequences.

In a surprising result, the pseudotyped viruses of the present invention were found to effectively transduce airway epithelium when introduced to the apical surface of the airways. One of the major barriers to gene therapy in airways is the resistance of airway epithelium to transduction by viral vectors *in vivo*. The apical surface of the epithelial is the surface that the viral vectors contact when provided directly into the airways. Multiple factors present on the apical surface of epithelia may act as physical barriers preventing vector access to receptors. These include mucus, airway surface liquid and its components, immune effector cells, such as macrophages and neutrophils, and the extracellular matrix. Although the viral vectors for transporting CFTR into airway epithelial have been constructed previously, none have been particularly effective.

In another embodiment, the method includes contacting, or transducing, a hepatocyte or brain glial cell with a lentivirus that has been pseudotyped with togaviral glycoproteins that includes the desired nucleotide sequence in its genome. The level of transduction may be monitored by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences. In a surprising result, the pseudotyped viruses of the present invention were found to effectively transduce hepatocytes *in vivo*. Previous reports of transduction of hepatocytes by retroviral vectors have suggested that *in vivo* gene therapy for liver defects and diseases would be difficult. Figures 1A-1F show that transduction with RRV-pseudotyped FIV is extensive throughout the liver (Figure 1A), especially when compared to a VSV-G pseudotyped lentivirus control (Figure 1B). Furthermore, the viruses of the present invention do not affect liver function as measured by SGOT and SGPT levels of treated livers (Figures 2A and 2B). This is in stark contrast to the VSV-G pseudovirus (Figures 2A and 2B), which has been reported to be toxic to a variety of cell types.

In an alternate embodiment, the cells are brain glial cells. One type of glial cell, oligodendrocytes, is responsible for formation of the myelin sheath that protects

the spinal cord. In multiple sclerosis, both oligodendrocytes and the myelin sheath are destroyed. Another type of glial cell, astrocytes, contains high affinity glutamate transporters that are critical in maintaining the extracellular glutamate concentration at sub-excitotoxic levels and thereby preventing neuronal cell death. Insufficient glutamate uptake by the transporters is believed to play a role in amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia, and AIDS by way of non-limiting example. Astrocytic uptake of glutamate may also serve to fine-tune the time course of glutamate in the synaptic cleft, perhaps by terminating the synaptic signal. Additionally, astrocytes may mediate inter-synaptic spillover of glutamate. The togaviral glycoprotein pseudotyped lentiviruses of the present invention are selective for transducing glial cells as compared to other CNS cells. A feline immunodeficiency virus (FIV) pseudotyped with at least two different Ross River (RRV) viral glycoproteins was effective in transducing brain astrocytes (Figures 3A-3C). The presence of the marker protein GFAP confirmed that the brain glial cells were astrocytes. The FIV virus pseudotyped with RRV glycoproteins was also effective in transducing oligodendrocytes (Figures 4A-4C and 5A-5C). The presence of the marker protein CNPase confirmed that the brain glial cells were oligodendrocytes. The data in the table of Figure 6 confirms the selective transduction of astrocytes and oligodendrocytes (oligos) by the togaviral pseudotyped lentivirus as compared to other types of brain cells.

The pseudotyped viruses can be introduced into a mammal requiring gene therapy by a number of ways known to the skilled artisan. For airway epithelium, the viruses can be introduced directly into the airway by inhalation aided by a nebulizer or an inhaler. The pseudotyped lentiviruses of the present invention can also be injected intravenously for systemic gene delivery. The pseudotyped lentiviruses can also be injected directly into the liver or the brain parenchyma. Alternatively, hepatocytes, brain glial cells or airway epithelial cells may be removed from the mammal, transduced with the pseudotyped lentiviruses and then implanted back into the patient.

The present invention also provides methods of screening agents effective in blocking viral entry into a cell. The methods allow for direct screening as the viral entry step can be detected in the method. In one embodiment, the method comprises treating the cell or the virus with the desired agent, contacting the cell with the virus,

and detecting viral entry into the cell. A wide variety of agents may advantageously be screened in the present invention, including, immunological agents such as monoclonal and/or polyclonal antibodies. Alternately, various pharmacological agents may also be screened in the present method in the same way, and may include proteins, peptides and various chemical agents.

In yet another embodiment, kits for forming inventive filovirus glycoprotein-pseudotyped lentivirus are provided. The kits contain the plasmids and nucleic acid sequences required to transform a cell to produce the desired virus.

The foregoing and other aspects of the invention may be better understood in connection with the following example, which is presented for purposes of illustration and not by way of limitation.

EXAMPLE

Methods for preparing and administering pseudotyped vectors to models:

Vector production. The second generation FIV vector system was previously reported. Johnston, J.C. et al, *J Virol.* 73:4991, (1999). Plasmid constructs consist of an FIV packaging construct with a deletion in the *env* gene and mutations in *vif* and *orf2*, an FIV vector construct expressing cytoplasmic *E. coli* β-galactosidase, eGFP or other nucleic acid sequences of interest, and an envelope plasmid in which the human CMV early gene promoter directs transcription of the Marburg envelope cDNA. The FIV packaging plasmid (pCFIVΔ*orf2*Δ*vif*) contains the FIV packaging signal (ψ), the *gag* and *pol* genes, and the *rev* sequences. FIV *rev* is analogous to the HIV *rev* in enabling expression of late genes encoded by unspliced or singly spliced mRNAs containing the cis-acting Rev-responsive element (RRE). The proviral FIV 5' LTR is replaced by the CMV promoter/enhancer and the 3' LTR is replaced with the simian virus 40 polyadenylation signal. A deletion in the *env* gene and mutations in FIV accessory genes *vif* and *orf2* render these sequences inactive without negatively affecting vector titer.

The FIV vector plasmids (based on $pVET_L$) consist of the FIV 5' and 3' LTR sequences flanking a portion of the *gag* sequence including the packaging signal, a transgene cassette, and the RRE. The U3 region of the 5' FIV LTR is replaced with the CMV promoter. A CMV promoter-β-Gal expression plasmid, pCMVβgal, was

generated by combining an *XbaI/SalI* fragment corresponding to the CMV promoter from pCMV-G and a *SalI/SmaI* fragment corresponding to the β-Gal gene from pSP6-β-GAL into pBlueScript SK(−). pTFIVLCβ, pTC/FLCβ, and pTC/FSCβ were then generated by insertion of the *NotI/SmaI* CMV-β-Gal expression cassette from pCMVβ gal into similarly digested pTFIVL, pTC/FL, and pTC/FS vector backbones, respectively. These constructs were renamed $pTFIV_LC\beta$, $pVET_LC\beta$, and $pVET_SC\beta$, respectively. A pCMVβgalCTE expression plasmid was used to generate an FIV expression vector containing the constitutive RNA transport element (CTE) from Mason-Pfizer monkey virus (MPMV). pCMVβgalCTE was constructed in part from pSK-CTE. pSK-CTE was generated by PCR amplification of the CTE with the primers CTEH5 and CTEH3, which harbor *Hin*dIII sites near their 5' ends. The resulting PCR product was digested with *Hin*dIII and inserted into similarly digested pBlueScript SK(−) to generate pSK-CTE. pSK-CTE was then digested with *Sma*I and *Xho*I, and the insert was ligated into similarly digested pCMV3gal to generate pCMVβ galCTE. A *NotI/Xho*I fragment containing the CMVβgalCTE expression cassette from pCMVβgalCTE was then ligated into *NotI/SalI*-digested pTC/FL to create pTC/FLCβ CTE (now referred to as $pVET_LC\beta_{CTE}$).

The VSV-G envelope plasmid, pCMV-G, encodes the VSV envelope glycoprotein. Yee, J.K. et al., *Proc. Natl. Acad. Sci. USA* 91: 9564-9568 (1994). The pRRV-E2E1 plasmid encodes the full-length RRV envelpe glycoprotein, E3-E3-6K-E1, which is processed proteolytically into the individual subunits. The region encoding the RRV envelope glycoproteins was amplified from pRR64, which contains the full-length cDNA of the RRV genome (Kuhn, R.H. et al., *Virology* 182: 430-441 (1991)), using *Taq* DNA polymerase (Promega Corporation) and two primers complementary to the viral cDNA at nucleotides 8376 and 11312. The amplified fragment, which contained the RRV E3-E2-6K-E1 coding region, was digested with the restriction endonucleases *Bam*HI and *Xba*I and ligated into the *Bam*HI and *Xba*I sites of pBacPac, a baculovirus expression vector (Clontech). The resulting plasmid was digested with *Bam*HI and *Xba*I, and the fragment containing the RRV E3-E2-6K-E1 coding region was ligated into the *Bam*HI and *Xba*I sites in the pcDNA3 and pcDNA3.1/Zeo(+) mammalian expression vectors (Invitrogen). The resulting plasmids were designated pRRV-E2E1 and pRRV-E2E1A, respectively.

To construct this plasmid, the nucleotides 5931-8033 from the Marburg virus genome (SEQ. ID. NO: 8, Genbank Accession Number Z12132) were cloned into the pSP72 plasmid (from Promega) under the control of the T7 promoter using SalI. The XhoI and Eco RI fragment of this plasmid was cloned into the XhoI and Eco RI polylinker sites of the mammalian expression vector pcDNA3. SEQ ID 9 also shows the amino acid sequence of the Marburg virus glycoprotein

Pseudotyped FIV vector particles were generated by transient transfection of plasmid DNA into 293T cells plated 1 day prior to transfection at a density of 2.8×10^6 per 10-cm-diameter culture dish as described by Johnston, J.C. et al, *J Virol.* 73:4991, (1999). Three plasmid cotransfections were performed using packaging, envelope, and vector plasmids, followed by collection of supernatants and particle concentration by centrifugation. For each preparation, 750 ml of culture supernatant was centrifuged overnight at 7,400 x g and resuspended in 3 ml of lactose buffer (19.5 mM Tris at pH 7.4, 37.5 mM NaCl, and 40 mg/ml lactose). Transduction titers before and after concentration were determined by measurement of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-positive cells in tranduced HT-1080 target cells and wre expressed as tranducing units (TU)/ml.

Gene transfer methods

Lung

In vitro. To transduce differentiated human epithelia, the pseudotyped FIV vector was mixed with cell culture medium to a final volume of 100 μl (MOI ~10). This mixture was applied to either the apical surface or the basal surface of primary cultures of human airway epithelia as described previously. Wang, G. et al., *J Virol.* 104: R49-R56, (1999). To enhance transduction from the apical surface, vector was mixed at a 1:1 (vol/vol) ratio with 12 mM EGTA HEPES/saline solution (pH 7.3), and applied apically for 4 hours as previously reported for Murine leukemia virus vectors. Wang, G. et al., *J Virol.* 104: R49-R56, (1999). The results are shown in Figure 1. The pseudotyped FIV vector was effective in transducing cells when applied to either the basal or apical surface of the cells. In contrast, the VSV-G control could not transduce the cells when applied to the apical surface.

In vivo. For tracheal gene transfer, adult New Zealand white rabbits are anesthetized with 32 mg/kg ketamine, 5.1 mg/kg xylazine and 0.8 mg/kg

acepromazine intramuscularly, a ventral midline incision made and tracheotomy performed. An approximately 1.5 cm tracheal segment cephalad to the tracheotomy was isolated and cannulated on each end with PE 330 tubing (Clay Adams, Becton Dickinson). The tracheal segment was rinsed and then filled with a FIV-β–gal vector solution. The vector solution was left in place for 45 min, then the cannulae were removed and the incisions closed. Five days or 6 weeks later, the tissues are studied for β-galactosidase expression. For lower airway gene transfer, a PE50 catheter was passed via the trachea until it lodged in a subsegmental bronchus. 200-600 μl of FIV-β–gal of various envelope pseudotypes was instilled. Five days later, the tissues are studied for β-galactosidase expression.

Brain

Six to 8 week old adult male C57BL/6 mice were used for gene transfer. Mice were anaesthetized and 5 x 10^5 TU of the vectors were stereotactically injected into either the right lateral ventricle or the right striatum, using a 26 gauge Hamilton syringe driven by a microinjector (Micro 1, World Precision Instruments, Sarasota, FL) at 0.5 μl per minute. For ventricular injections, 10 μl volumes were injected at coordinates 0.4 mm anterior, 1.0 mm lateral to bregma at 2 mm depth. For striatal injections, 5 μl volumes are injected at coordinates 0.4 mm rostral and 2 mm lateral to bregma, and at a 3 mm depth. A minimum of two independent experiments are done for each vector and injection site. At 3 weeks postinjection, mice were sacrificed and perfused with 2% formaldehyde in PBS. The brains were postfixed overnight at 4 °C and cryoprotected in 30% sucrose-PBS for 48 h at 4 °C. The hemispheres were separated and blocked in O.C.T. (Sakura Finetek USA, Torrance, CA) by freezing in a dry ice-ethanol bath. Parasagittal cryosections (10 μm) were cut and placed on slides. Slides were stained with X-Gal or were dually stained with antibodies for immunofluorescent confocal analysis.

Liver

The C57Bl/6 mice were intravenously injected via tail vein with FIV vector (total dose 1.3x10^7 to 6x10^7 IU), administrated over one or on two consecutive days (one injection/day). Controls received vector buffer. The injection volume was 0.4

ml. On days 1 and 7 postinjection, blood samples were obtained from the retro-orbital plexus and the serum samples assayed for the levels of glutamic oxalacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) using a transaminase assay kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. At 3 weeks postinjection, the mice were sacrificed and perfused with cold phosphate-buffered saline (PBS). Samples of liver, spleen, kidney, lung, heart, and skeletal muscles (triceps) were harvested for X-Gal staining.

Determination of β-galactosidase expression

For X-Gal staining of liver after intravenous vector injection, lobes were fixed in 2% paraformaldehyde-PBS overnight and then stained with X-Gal overnight at 4 °C. The overall expression of b-galactosidase was first examined by stereo microscopy. The X-Gal-stained tissue was then embedded in paraffin, and 5-μm sections were cut at 50-μm intervals and counterstained with hematoxylin and eosin for quantification and histological examination. For X-Gal staining of brain and muscle sections, 10-mm sections on slides were incubated in X-Gal for 6 h at 37 °C, washed in PBS, and counterstained with neutral red. For X-Gal staining of lung, the lungs were removed, inflated with and submersed in 2% paraformaldehyde-PBS, and allowed to fix for 4 h at 4 °C. After fixation, the lungs were washed with PBS and inflated with X-Gal solution. The lungs were submersed in additional X-Gal and incubated overnight at 37 °C. After X-Gal staining, the lungs were washed with PBS and paraffin embedded by a standard protocol, and 10-μm sections were collected. Sections were counterstained with nuclear fast red.

Immunostaining

To determine the cell types transduced after intrastriatal injections of RRV pseudotyped FIV, 10-μm brain sections were dually stained for β-galactosidase and glial fibrillary acidic protein (GFAP, a type II astrocyte-specific intermediate filament), or for β-galactosidase and 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase, an oligodendrocyte-myelin specific marker) and analyzed by confocal fluorescence microscopy. The antibodies used were polyclonal rabbit anti-β-galactosidase (Biodesign International, Saco, ME), Cy3-conjugated mouse

monoclonal anti-GFAP (Sigma), mouse monoclonal anti-CNPase (Sigma) Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and lissamine-rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Sections were blocked with 10% normal goat serum and 0.1% Triton X-100 in PBS for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C in PBS with 3% bovine serum albumin and 0.1% Triton X-100. The sections were then washed, incubated with secondary antibodies for 2 h at room temperature, washed, and coverslipped with gel mount. Using confocal microscipy, images from 0.3-μm-thick Z series were collected.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

What is claimed is:

1. A pseudotyped lentivirus comprising:

a lentiviral capsid;

a lipid bilayer wherein said bilayer surrounds said capsid; and

a Marburg glycoprotein disposed in said lipid bilayer wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

2. The lentivirus of claim 1 further comprising a nucleic acid sequence encoding a desired protein, said nucleic acid sequence enclosed within said lentiviral capsid.

3. The lentivirus of claim 2 wherein the desired protein is CFTR.

4. The lentivirus of claim 1 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

5. A method of introducing a nucleic acid sequence encoding a desired protein into an airway epithelial cell comprising the step of transducing an airway epithelial cell with a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, a filoviral glycoprotein disposed in said bilayer and a nucleic acid sequence encoding a desired protein.

6. The method of claim 5 wherein said filoviral glycoprotein is a Marburg glycoprotein.

7. The method of claim 6 wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

8. The method of claim 5 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

9. The method of claim 5 wherein the desired protein is CFTR.

10. A method of introducing a nucleotide sequence encoding a desired protein into a hepatocyte or brain glial cell comprising the step of transducing a hepatocyte or brain glial cell with a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, at least two different togaviral glycoproteins disposed in said bilayer and a nucleotide sequence encoding a desired protein.

11. The method of claim 10 wherein said togaviral glycoproteins are alphaviral glycoproteins.

12. The method of claim 11 wherein the alphaviral glycoproteins are Ross River alphaviral glycoproteins.

13. The method of claim 10 wherein the retroviral capsid is comprised of a feline immunodeficiency virus capsid.

14. The method of claim 10 wherein the desired protein is the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter.

15. A method for introducing a nucleic acid sequence encoding a desired protein into the airway epithelial cells of a mammal comprising the step of administering to the lungs of the mammal a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, a filoviral glycoprotein disposed in said bilayer and a nucleic acid sequence encoding a desired protein wherein the nucleic acid sequence is enclosed within the lentiviral capsid.

16. The method of claim 15 wherein said filovirus glycoprotein is a Marburg glycoprotein.

17. The method of claim 16 wherein the Marburg glycoprotein has the mutation of C671A, F676stop or Y679stop.

18. The method of claim 15 wherein the lentiviral capsid comprises a feline immunodeficiency virus capsid.

19. The method of claim 15 wherein the desired protein is CFTR.

20. A method for introducing a nucleotide sequence encoding a desired protein into the liver or brain of a mammal comprising the step of administering to the mammal a pseudotyped lentivirus comprising a lentiviral capsid, a lipid bilayer wherein said lipid bilayer surrounds said capsid, at least two different togaviral glycoproteins disposed in said bilayer and a nucleotide sequence encoding a desired protein wherein the nucleotide sequence is enclosed within the lentiviral capsid.

21. The method of claim 20 wherein said togaviral glycoproteins are alphaviral glycoproteins.

22. The method of claim 21 wherein the alphaviral glycoproteins are Ross River alphaviral glycoproteins.

23. The method of claim 20 wherein the lentiviral capsid is comprised of a feline immunodeficiency virus capsid.

24. The method of claim 20 wherein the desired protein is the LDL receptor, alpha1-antitrypsin, ornithine transcarbamylase, Factor VIII or a high affinity glutamate transporter.

25. The method of claim 20 wherein the pseudotyped virus is administered to the mammal intravenously.

26. The method of claim 20 wherein the pseudotyped virus is administered by injection directly into the liver or brain.

ABSTRACT OF THE DISCLOSURE

Methods for introducing nucleic acid sequences into hepatocytes, brain glial cells and airway epithelial cells are provided. The methods use filoviral and togaviral glycoprotein-pseudotyped lentiviruses. The viruses comprise a lentiviral capsid and a viral envelope further comprising a lipid bilayer and a functional filoviral glycoprotein or two functional togaviral glycoproteins. In one embodiment the lentivirus is a feline immunodeficiency virus (FIV). In an alternate embodiment the filoviral glycoprotein is a Marburg virus glycoprotein. In another embodiment, the Marburg virus glycoprotein can have a mutation in the C-terminal portion of the amino acid sequence that results in a higher titer production of the pseudotyped virus. In an alternate embodiment the togaviral glycoproteins are alphavirus glycoproteins, for example, the E1 and E2 envelope glycoproteins of Ross River virus (RRV)

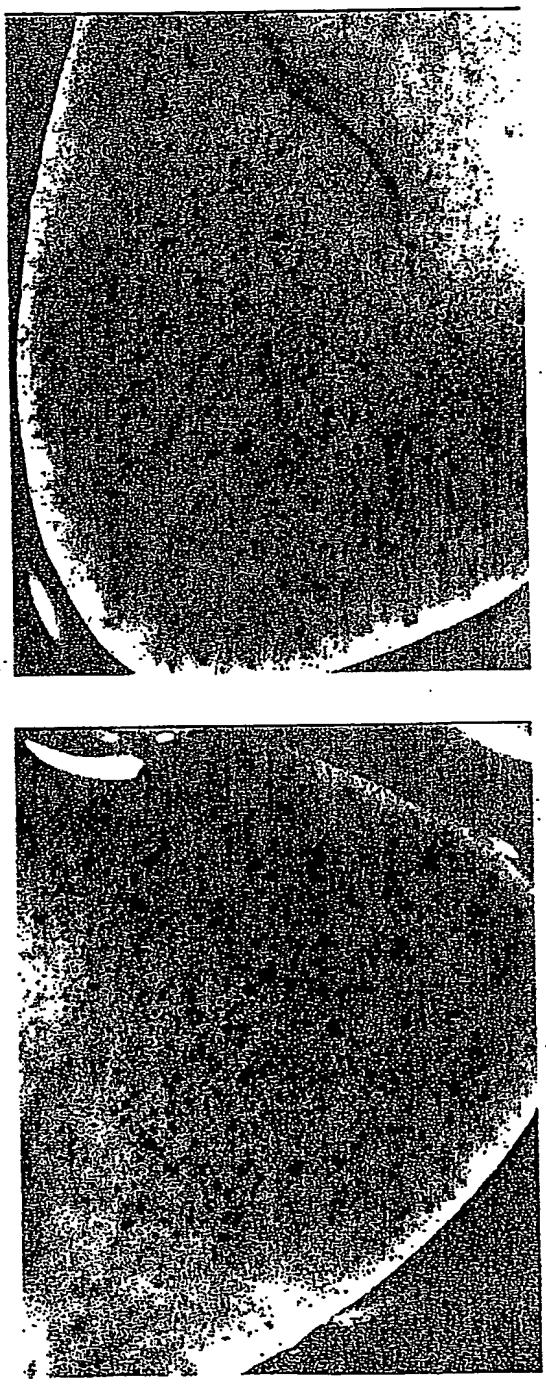


Fig. 1A

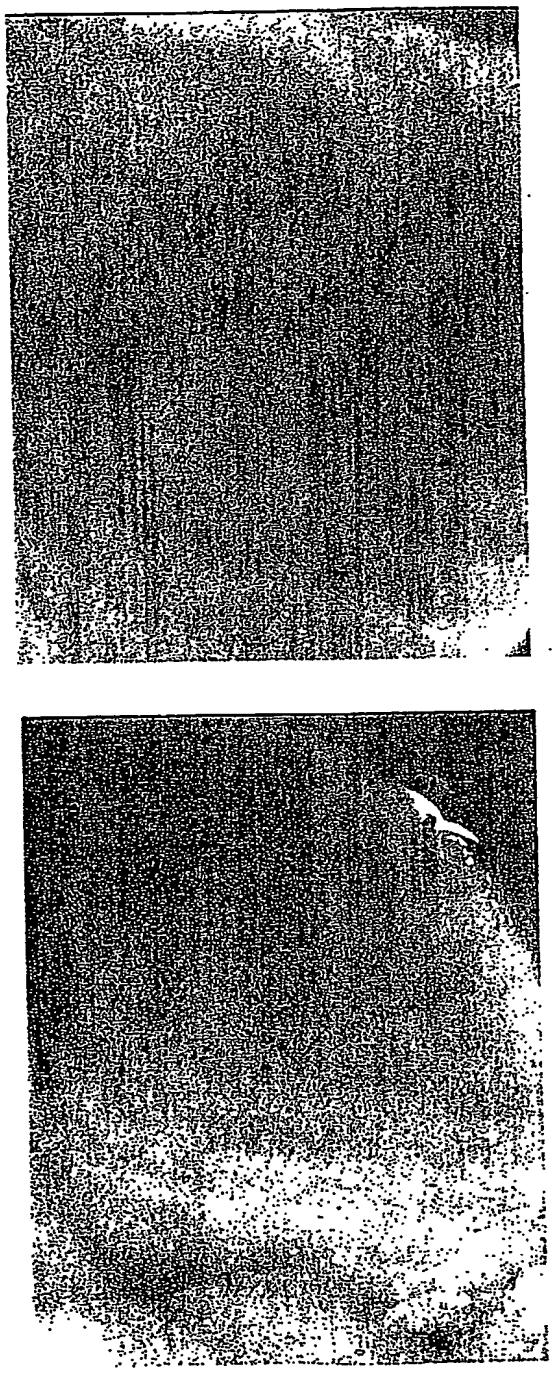


Fig. 1B

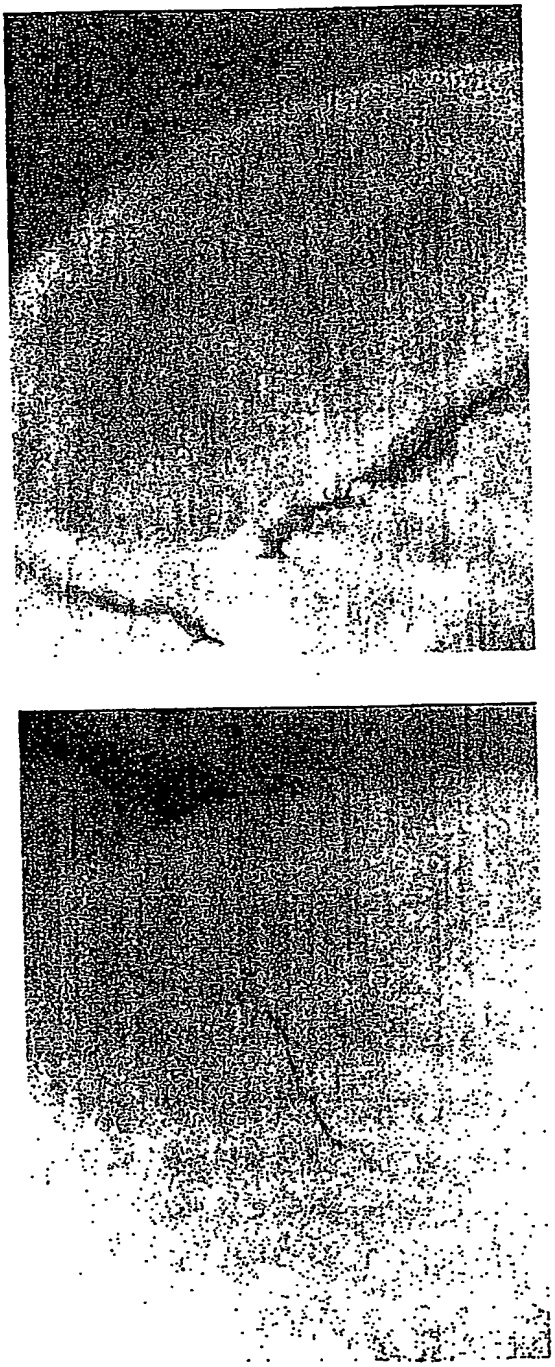


Fig. 1C

Fig. 1E

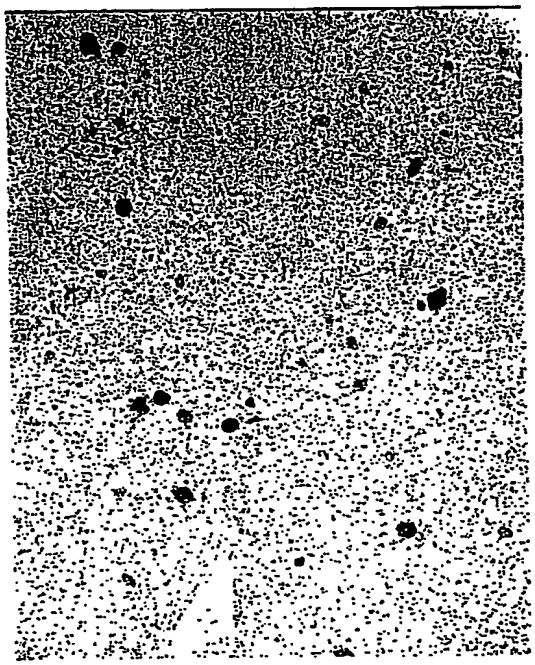


Fig. 1D

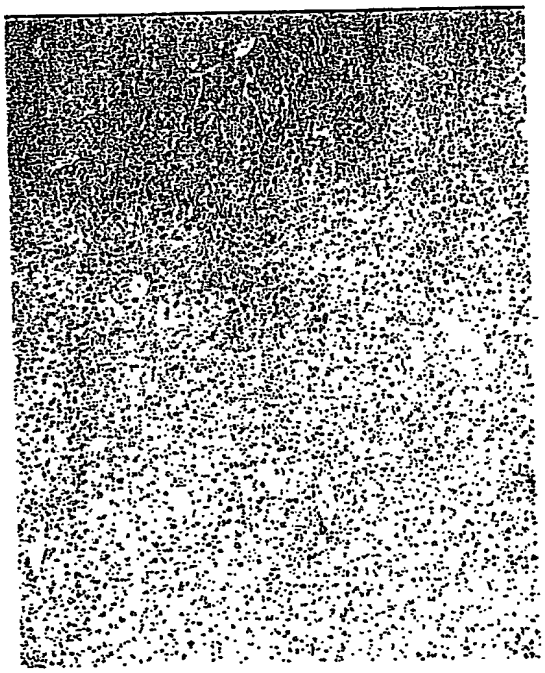


Fig. 1F

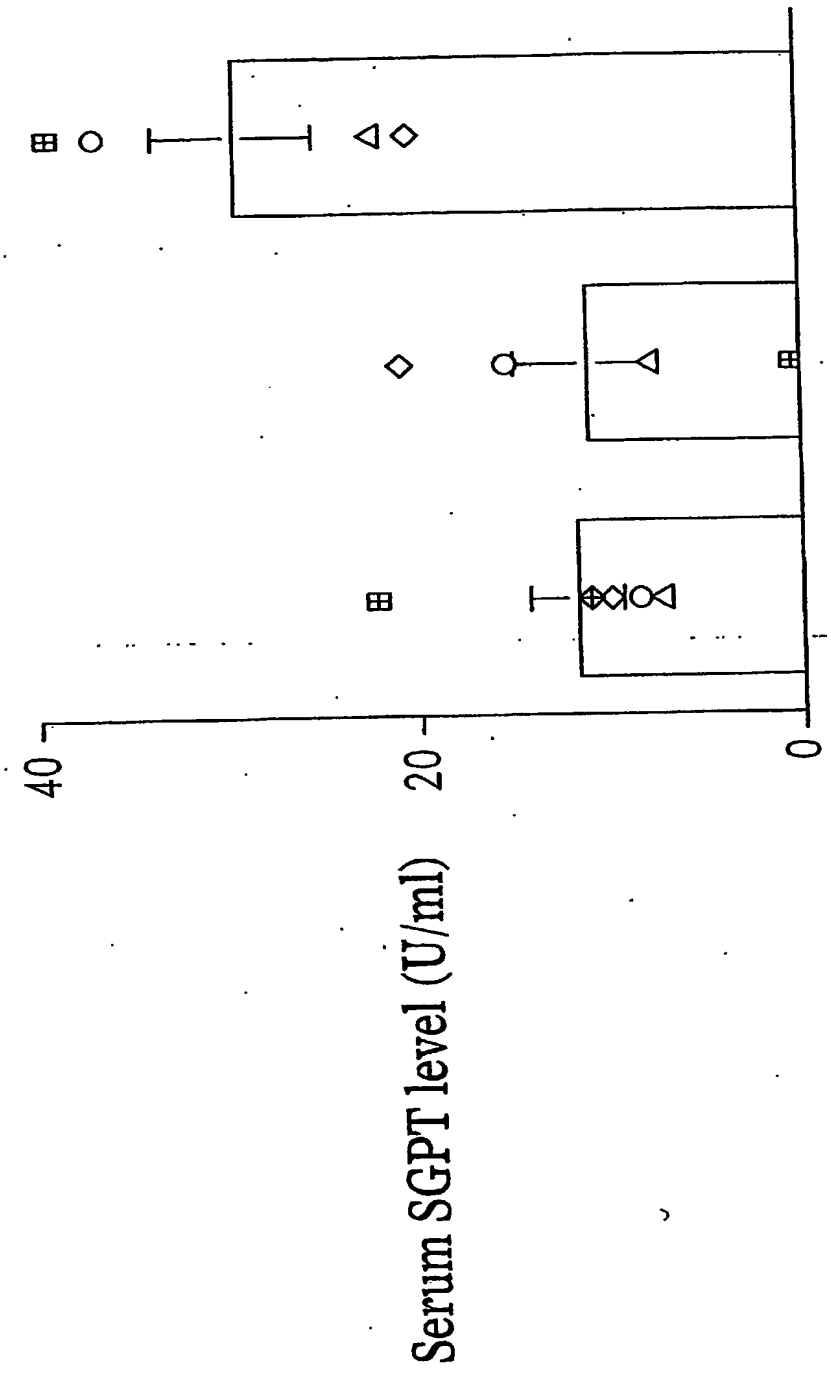


Fig. 2A

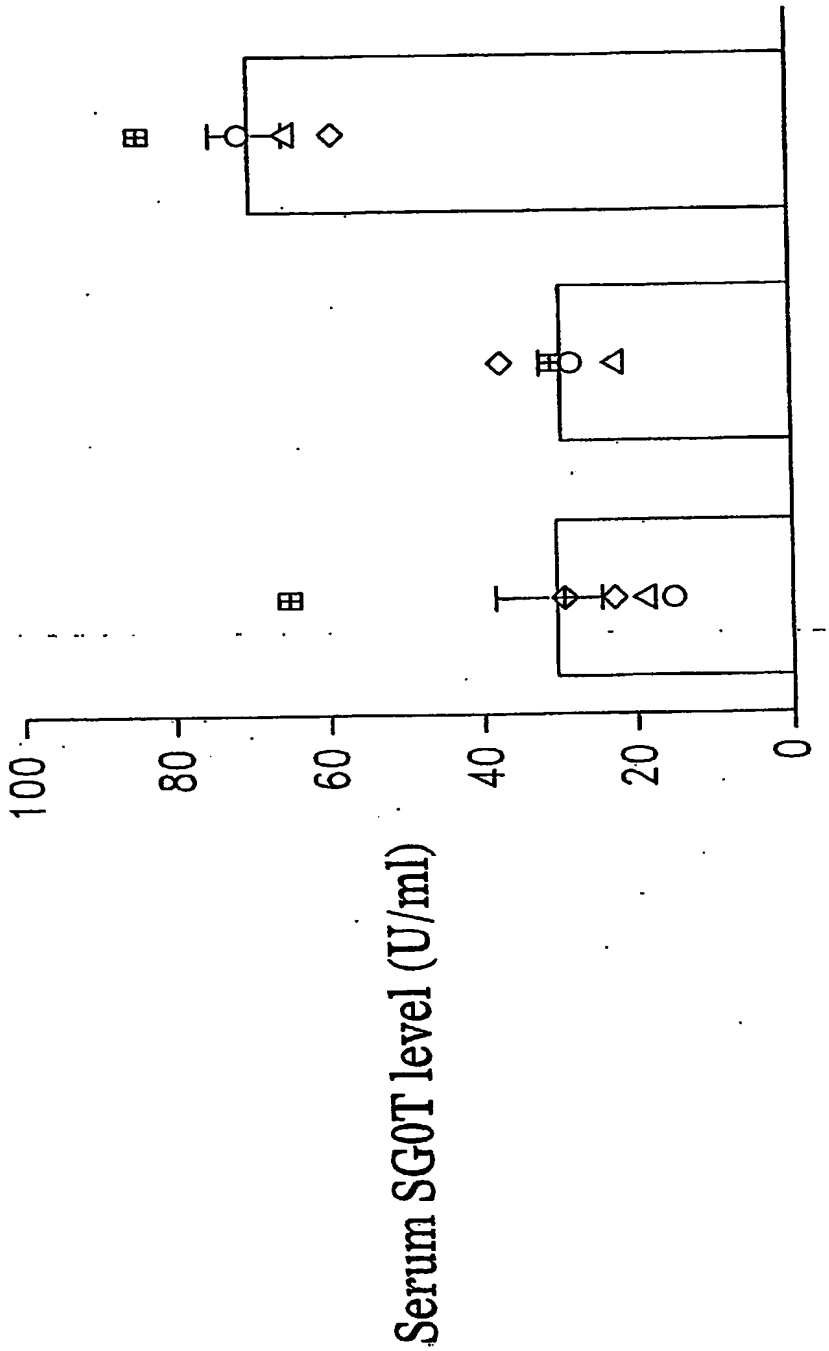


Fig. 2B

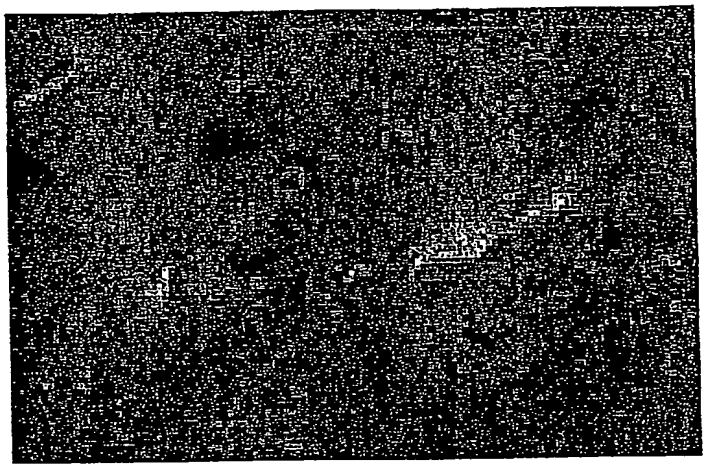


Fig. 3C

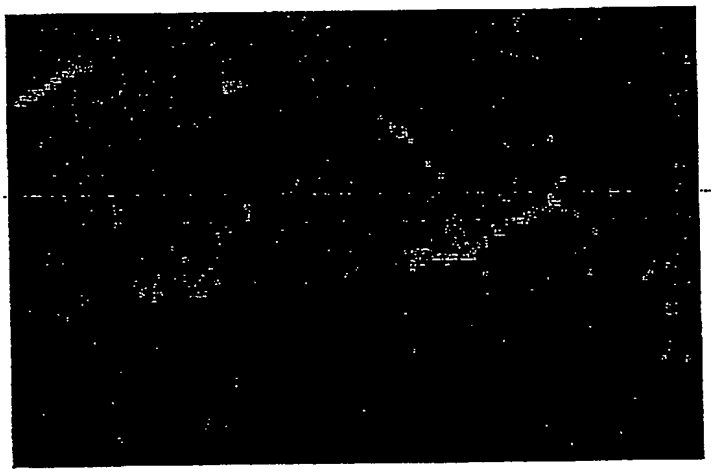


Fig. 3B

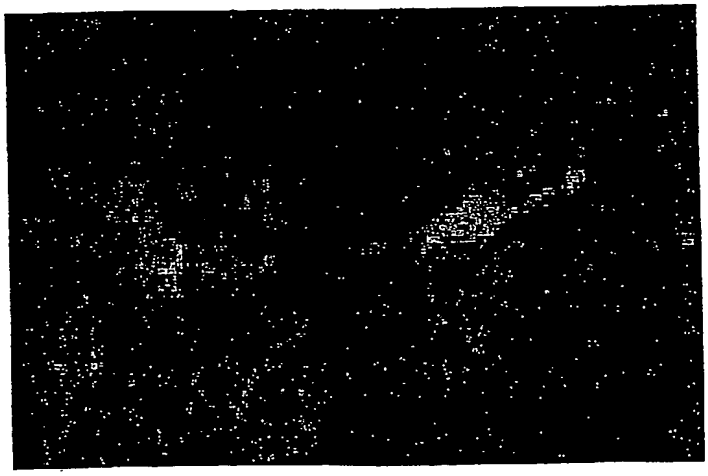


Fig. 3A

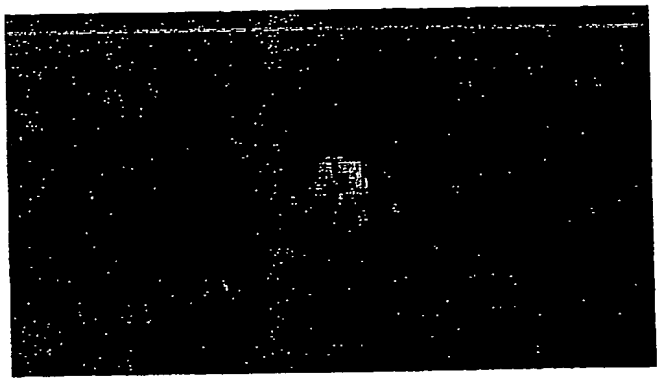


Fig. 4A

Fig. 4B

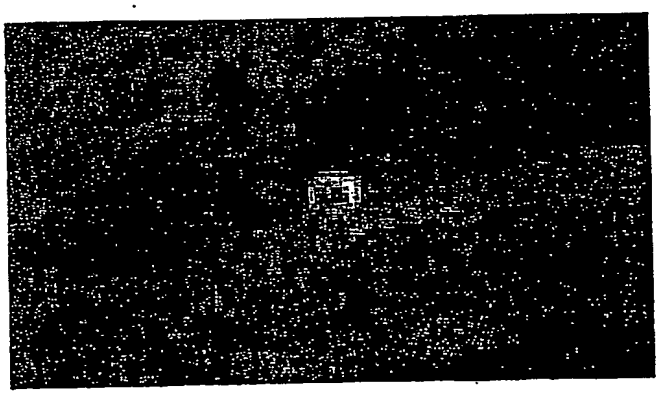


Fig. 4C

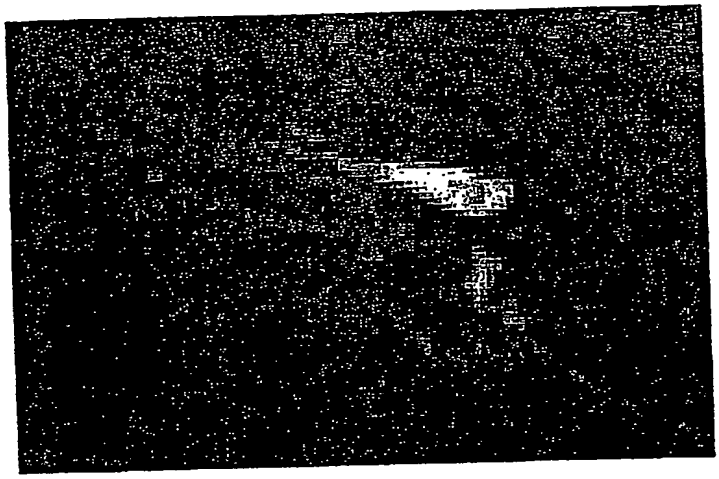


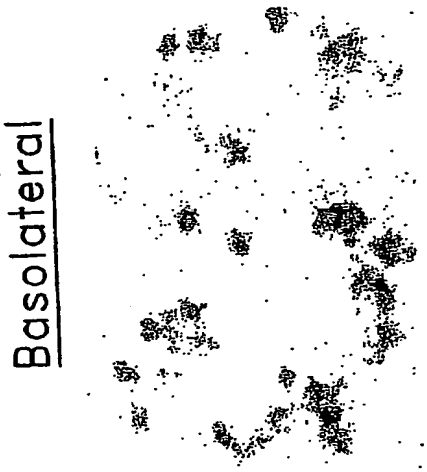
Fig. 5C

Fig. 5B

Fig. 5A

Cell Type	% Transduced
Neurons	7.0±6.5%
Astrocytes	56.5±17.2%
Microglia	9.9±5.5%
Oligos	26.6%

Fig. 6



Basolateral

Fig. 7B

Fig. 7D

Apical

Fig. 7A

Fig. 7C

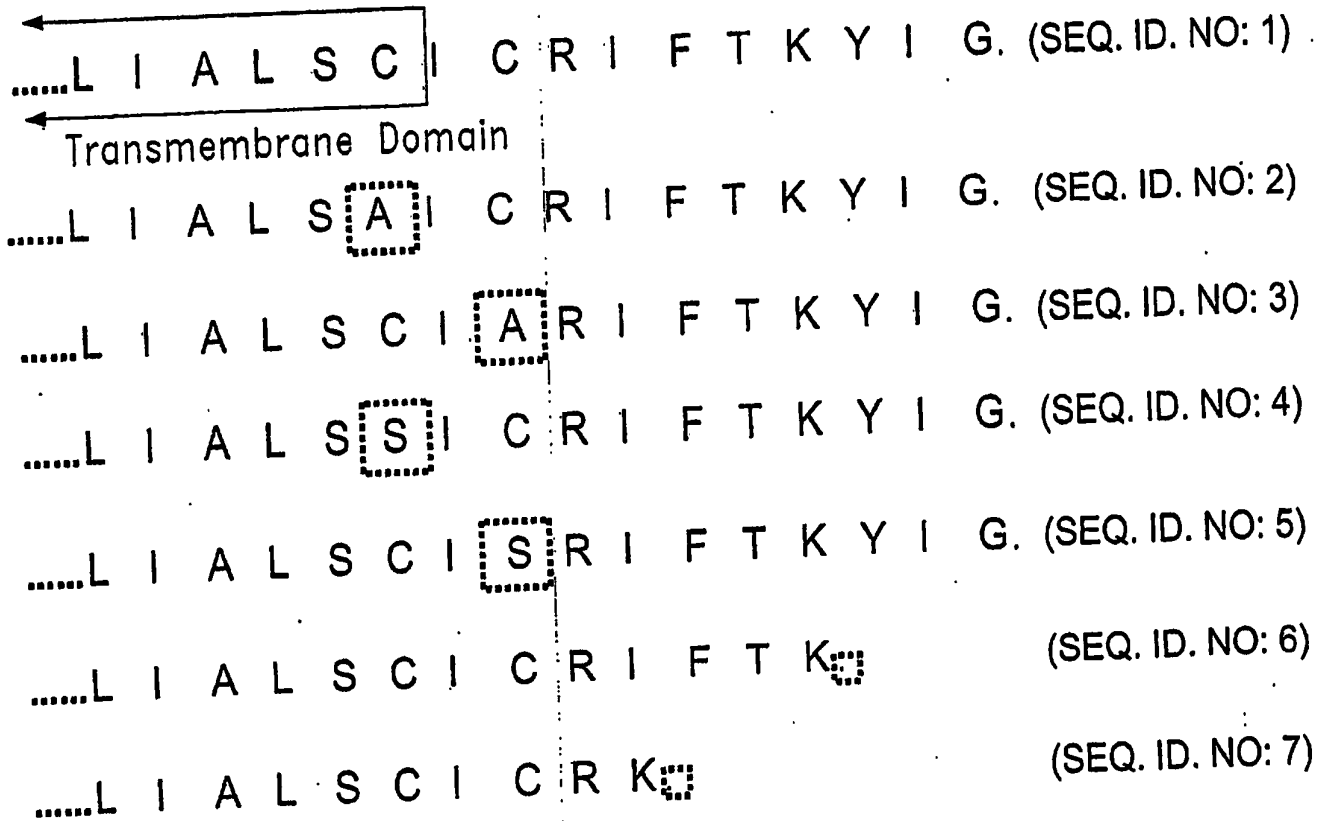

.....L I A L S C I C R I F T K Y I G. (SEQ. ID. NO: 1)
Transmembrane Domain
C671A .....L I A L S A I C R I F T K Y I G. (SEQ. ID. NO: 2)
C673A .....L I A L S C I A R I F T K Y I G. (SEQ. ID. NO: 3)
C671S .....L I A L S S I C R I F T K Y I G. (SEQ. ID. NO: 4)
C673S .....L I A L S C I S R I F T K Y I G. (SEQ. ID. NO: 5)
Y679Stop .....L I A L S C I C R I F T K□ (SEQ. ID. NO: 6)
Y676Stop .....L I A L S C I C R K□ (SEQ. ID. NO: 7)

Fig. 8

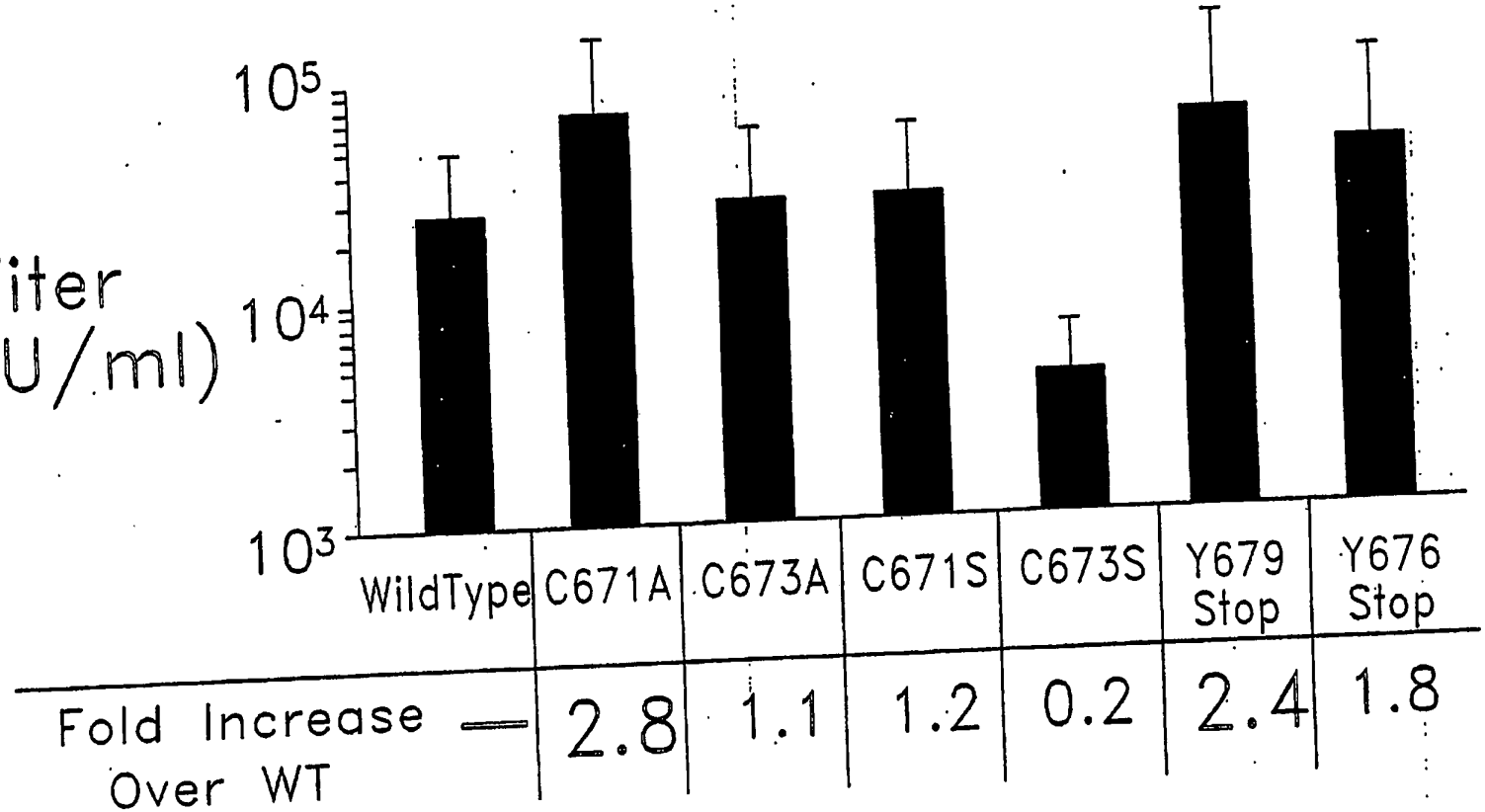

Titer (TU/ml)
10^5
10^4
10^3
WildType
C671A
C673A
C671S
C673S
Y679 Stop
Y676 Stop
Fold Increase Over WT
2.8
1.1
1.2
0.2
2.4
1.8

Fig. 9

1	MKTTCFLISL	ILIQGTKNLP	ILEIASNNQP	QNVDSVCSGT	LQKTEDVHLM
51	GFTLSGQKVA	DSPLEASKRW	AFRTGVPPKN	VEYTEGEEAK	TCYNISVTDP
101	SGKSLLLDPP	TNIRDYPKCK	TIHHIQGQNP	HAQGIALHLW	GAFFLYDRIA
151	STTMYRGKVF	TEGNIAAMIV	NKTVHKMIFS	RQGQGYRHMN	LTSTNKYWTS
201	SNGTQTNDTG	CFGALQEYNS	TKNQTCAPSK	IPPPLPTARP	EIKLTSTPTD
251	ATKLNTTDPS	SDDEDLATSG	SGSGEREPHT	TSDAVTKQGL	SSTMPPTPSP
301	QPSTPQQGGN	NTNHSQDAVT	ELDKNNTTAQ	PSMPPHNTTT	ISTNNTSKHN
351	FSTLSAPLQN	TTNDNTQSTI	TENEQTSAPS	ITTLPPTGNP	TTAKSTSSKK
401	GPATTAPNTT	NEHFTSPPPT	PSSTAQHLVY	FRRKRSILWR	EGDMFPFLDG
451	LINAPIDFDP	VPNTKTIFDE	SSSSGASAEE	DQHASPNISL	TLSYFPNINE
501	NTAYSGENEN	DCDAELRIWS	VQEDDLAAGL	SWIPFFGPGI	EGLYTAVLIK
551	NQNNLVCRLR	RLANQTAKSL	ELLLRVTTEE	RTFSLINRHA	IDFLLTRWGG
601	TCKVLGPDCC	IGIEDLSKNI	SEQIDQIKKD	EQKEGTGWGL	GGKWWTSDWG
651	VLTNLGILLL	LSIAVLIALS	CICRIFTKYI	G	

Fig. 10

Lentivirus Vectors Pseudotyped with Filoviral Envelope Glycoproteins Transduce Airway Epithelia from the Apical Surface Independently of Folate Receptor Alpha

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The practical application of gene therapy as a treatment for cystic fibrosis is limited by poor gene transfer efficiency with vectors applied to the apical surface of airway epithelia. Recently, folate receptor alpha (FRα), a glycosylphosphatidylinositol-linked surface protein, was reported to be a cellular receptor for the filoviruses. We found that polarized human airway epithelia expressed abundant FRα on their apical surface. In an attempt to target these apical receptors, we pseudotyped feline immunodeficiency virus (FIV)-based vectors by using envelope glycoproteins (GPs) from the filoviruses Marburg virus and Ebola virus. Importantly, primary cultures of well-differentiated human airway epithelia were transduced when filovirus GP-pseudotyped FIV was applied to the apical surface. Furthermore, by deleting a heavily O-glycosylated extracellular domain of the Ebola GP, we improved the titer of concentrated vector severalfold. To investigate the folate receptor dependence of gene transfer with the filovirus pseudotypes, we compared gene transfer efficiency in immortalized airway epithelium cell lines and primary cultures. By utilizing phosphatidylinositol-specific phospholipase C (PI-PLC) treatment and FRα-blocking antibodies, we demonstrated FRα-dependent and -independent entry by filovirus-pseudotyped FIV-based vectors in airway epithelia. Of particular interest, entry independent of FRα was observed in primary cultures of human airway epithelia. Understanding viral vector binding and entry pathways is fundamental for developing cystic fibrosis gene therapy applications.

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Fn* Viral vector-mediated gene transfer to airway epithelial cells as therapy for diseases such as cystic fibrosis (CF) presents many challenges. The pulmonary epithelia and resident immune effector cells possess innate and adaptive defenses that evolved to prevent the invasion of microbes; these same defenses may act as barriers for gene transfer vectors (27). In addition, Moloney leukemia virus-based retroviral vectors are hampered by the low proliferation rate of adult airway epithelial cells (26). In an effort to overcome adverse immune responses to vector-encoded proteins and the transient nature of gene expression with nonintegrating vector systems, we utilize a vector system based on the nonprimate lentivirus, feline immunodeficiency virus (FIV) (28, 29).

The apical surface of airway epithelia is notably resistant to gene transfer with several vector systems and therefore presents additional challenges for CF gene therapy. This obstacle is generally attributed to the basolateral polarization of the receptors for several classes of viral vectors. For example, the receptors for serotype 2 and serotype 5 adenovirus (CAR) and

AQ: B AAV-2 (heparin sulfate proteoglycan) are predominantly expressed on the basolateral surface of airway epithelia (6, 25). In the case of enveloped viruses, the glycoproteins bind to specific receptors on the cell surface to initiate membrane fusion; these envelope-receptor interactions dictate cellular tropism. Furthermore, the receptors for many commonly used retroviral envelopes appear to be functionally expressed basolaterally in polarized epithelia (4). To overcome these barriers to gene transfer, an improved understanding of receptor biology and virus-cell interactions is essential. There have been significant advances in the understanding of encapsidated virus-receptor interactions; however, the cellular receptors for many of envelope glycoproteins available to pseudotype lentiviral vectors are unknown or poorly characterized.

Filoviral envelope glycoproteins have received attention as candidates for pseudotyping retrovirus to target a variety of cell types (31). Together Ebola virus (EBO) and Marburg virus (MRB) comprise the two members of the viral family *Filoviridae*. In contrast to other enveloped RNA viruses such as paramyxoviruses, both retroviruses and filoviruses have a single type 1 transmembrane structural protein that assembles into homotrimers and mediates both receptor binding and fusion (30). Sequence analysis suggests an evolutionary relationship between the envelope glycoproteins of filoviruses and retroviruses (19, 20, 30), with evidence that filoviruses can infect the host through an airborne mechanism (10). However, the origins of the viruses or how they are maintained in nature is presently unknown.

Interestingly, recent studies suggest that the folate receptor alpha (FRα) directs cellular entry of retroviruses pseudotyped with filoviral envelope glycoproteins (2). FRα, a glycosylphosphatidylinositol (GPI)-linked protein, was identified as a potential receptor for filoviral glycoproteins through utilization of an expression library in cells nonpermissive for viral entry. In Jurkat cells, FRα expression facilitated MRB- or EBO-pseu-

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dotyped Moloney leukemia virus entry. In addition, FR-blocking reagents inhibited transduction of HOS or HeLa cells (2). These data suggest that FRα provides one cellular entry pathway for wild-type filovirus as well as retroviruses pseudotyped with filoviral glycoproteins. Recently, the feasibility of pseudotyping human immunodeficiency virus-based lentiviral vectors with filovirus envelope glycoproteins to target airway epithelia has been demonstrated (14); however, FRα-dependent entry has not been investigated in this important model system.

Herein we report the expression of FRα in airway epithelia and the polarity of FRα expression in well-differentiated primary cultures of human airway epithelia. In addition, we investigate the role of FRα as a receptor for FIV-based lentivirus pseudotyped with filoviral envelope glycoproteins in airway epithelial cells.

MATERIALS AND METHODS

Culture of human airway epithelia. Airway epithelia were isolated from trachea or bronchi and were grown at the air-liquid interface as described previously (13). All preparations used were well differentiated (>2 weeks old; resistance > 1,000 Ω-cm^2). This study was approved by the Institutional Review Board at the University of Iowa. A549 and H441 cell lines are derived from human lung carcinomas, and IB3 and HBE cell lines are transformed human airway cells. The cell lines HT1080 (ATCC 12012), HOS (ATCC CRL-1543), IB3 (34), and KB (ATCC CCL-17) were maintained in Dulbecco's modified Eagle's medium (Gibco)–10% fetal bovine serum (FBS). A549 (ATCC CCL-185) cells were maintained in Dulbecco's modified Eagle's medium F12 (Gibco) [illegible] FBS. H441 (ATCC HTB-174) cells were maintained in RPMI medium (Gibco) [illegible] FBS. HBE (5) cells were maintained in modified Eagle's medium (Gibco) [illegible] FBS. In addition, each medium was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). In the FRα-blocking studies, the cells were washed [illegible] and maintained 72 h in RPMI medium lacking folic acid (Gibco; 27016-024) and 5% FBS prior to the addition of the blocking reagent.

Vector production. The second-generation FIV vector system utilized in this study was reported previously (11, 29). The FIV vector construct expressed the β-galactosidase cDNA directed by the cytomegalovirus promoter. All of the envelope constructs reported in this study utilized the cytomegalovirus early gene promoter to direct transcription. Those envelopes include the vesicular stomatitis virus G protein (VSVG), the EBO (Zaire strain) envelope glycoprotein (pEZGP [9]), and the MRB (Musoke variant) envelope glycoprotein (pMBGP [30]). EBOΔO (pEZGP 309-489) has been previously described (9). All of the filoviral envelope constructs reported in this study were expressed from pcDNA3.1 (Invitrogen, Carlsbad, Calif.)-derived plasmids. Pseudotyped FIV vector particles were generated by transient transfection of plasmid DNA into 293T cells as described previously (11). FIV vector preparations were titered on HT1080 cells at limiting dilutions, and these titers were used to calculate the multiplicities of infection (MOIs). In addition, we found that the filoviral glycoprotein conferred enough stability to the lentiviral vector to withstand centrifuge concentration of greater than 1,000-fold (data not shown); however, we typically concentrated vector 250-fold by centrifugation for in vitro experiments.

RPA. FRα mRNA levels were determined by RNase protection assay (RPA) as previously described (23). The FRα probe was a partial cDNA sequence cloned into pCR2.1-TOPO (Invitrogen). The human β-actin cDNA templates were obtained from Ambion (Austin, Tex.). The full-length probe for FRα and human actin were 541 and 315 bp, respectively. The expected protected fragment sizes were 413 and 245 bp, respectively. The RPA reaction was conducted by using an RPA III kit with the manufacturer's protocol (Ambion) and was quantified with a Molecular Dynamics Storm 620 PhosphorImager System and the ImageQuant software provided by the manufacturer.

FACS. For fluorescence-activated cell sorter (FACS) analysis, approximately 10^6 cells were first incubated in suspension with FcγII αCD32-blocking antibody (14531; Stem Cell Technologies) on ice for 15 min. Then a monoclonal antibody against FRα (MOv18; a kind gift from Silvana Canevari [18]) or the appropriate immunoglobulin G1 (IgG1) isotype control (554121; Pharmingen) was added and incubated on ice for 30 min. Cells were washed three times with 3% FBS in 1× phosphate-buffered saline (PBS). A goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated 2° antibody (31569; Pierce) was then added and again incubated on ice for 30 min. Finally, cells were washed as before and resuspended in 500 μl of 3% FBS in 1× PBS. Data were collected by using a FACScan flow cytometer (Becton Dickinson), and the data were analyzed using CellQuest software.

Western blot analysis. Western blot analysis for verifying FRα protein expression was conducted by using standard techniques. Briefly, cell lysates were denatured for 5 min at 100°C in Laemmli sample buffer, electrophoresed on 10% polyacrylamide gels (161-1155; Bio-Rad) at 125 V, and transferred to pure nitrocellulose (162-0145; Bio-Rad) overnight at 200 mA. The membrane was probed with a monoclonal anti-human-FRα primary antibody (MOv18; 3 mg/ml) at 1:1,000 and was detected by using goat anti-mouse IgG conjugated to alkaline phosphatase at a 1:1,000 dilution (A-1682; Sigma).

Immunohistochemistry and confocal microscopy. Epithelial cells were rinsed with 1× PBS, fixed in 2% paraformaldehyde for 5 to 10 min, and rinsed with 1× PBS. The epithelial cells were then incubated for 30 min at 37°C with a monoclonal anti-human FRα antibody (MOv18) or the appropriate isotype control diluted 1:100 in Hank's buffer (Gibco). The cells were washed with 1× PBS and were incubated with an FITC-conjugated anti-mouse 2° antibody (F-4143; Sigma) diluted 1:100 in 1× PBS for 30 min at 37°C. The primary and secondary antibodies were always applied to both the apical and basolateral surfaces of nonpermeabilized cells. Images were captured with a Bio-Rad MRC-1024 Hercules laser scanning confocal microscope equipped with a Kr/Ar laser.

Viral vector administration. Pseudotyped FIV vector was applied directly to immortalized cell lines for 4 h at 37°C. Following incubation with the vector, cells were rinsed in media and cultured for 4 days. Following the 4-day incubation, cells were harvested and β-galactosidase activity was quantified. Primary cultures of human airway epithelial cells were transduced with pseudotyped FIV vector by diluting vector preparations in media to achieve the desired MOI, and 100 μl of the solution was applied to the apical surface of airway epithelial cells. After incubation for 4 h at 37°C, the virus was removed and cells were further incubated at 37°C for 4 days. To infect airway epithelia with pseudotyped FIV vector from the basolateral side, the Millicell culture insert containing the airway epithelia was turned over and the virus was applied to the basolateral surface for [illegible] μl of media. Following the 4-h infection, the virus was removed and the culture insert was turned upright and allowed to incubate at 37°C, 5% CO_2, for [illegible].

β-Galactosidase quantification and AZT administration. The Galacto-light chemiluminescent reporter assay (Tropix, Bedford, Mass.) was used to quantify β-galactosidase activity following the manufacturer's protocol. The relative light units were quantified with a luminometer (Monolight 3010; Pharmingen) and were standardized to total protein as determined by modified Lowry assay (2024; Pierce Biotechnology) by using the manufacturer's protocol. To verify that the β-galactosidase activity observed in the transduced cells was due to reverse transcription-dependent expression and not the result of pseudotransduction of β-galactosidase present in the vector preparations, cells were infected in the presence or absence of zidovudine (AZT). The cells were incubated with 50 μM AZT (GlaxoWellcome) for 24 h prior to infection and were maintained in the media following vector administration.

Administration of FRα blockers. To cleave GPI-linked cell surface proteins, cells were pretreated with 2 U of phosphatidylinositol-specific phospholipase C (PI-PLC) (P-6466; Molecular Probes)/ml for 2 h at 37°C. Following enzyme treatment, viral vector challenge and β-galactosidase detection proceeded as described above. To specifically block FRα, cells were preincubated with a mouse monoclonal FRα-blocking antibody (IgG1) (HFBP 458; a generous gift of Wilbur Franklin [7]) or an isotype control antibody (554121; Pharmingen) for 15 min at room temperature. We diluted the purified blocking antibody or isotype control antibody 1:100 in USG (2-μg/ml final concentration). Following antibody treatment, viral vector challenge and β-galactosidase detection proceeded as described above.

Statistics. Unless otherwise noted, all numerical data are presented as the mean plus or minus standard deviation. Statistical analysis was performed with a two-tailed, unpaired Student *t* test by using Microsoft Excel software.

RESULTS

Expression of FRα in primary cultures of human airway epithelial cells. The identification of FRα as a mediator of filovirus cell entry offers the ability to investigate virus-host cell receptor interactions and pathways of infection. Chan and colleagues observed that PI-PLC and FRα antiserum inhibited entry of retrovirus pseudotyped with filoviral glycoproteins in a select group of cell types; however, the authors acknowledged that FRα may not facilitate virus entry into all cell types (2).

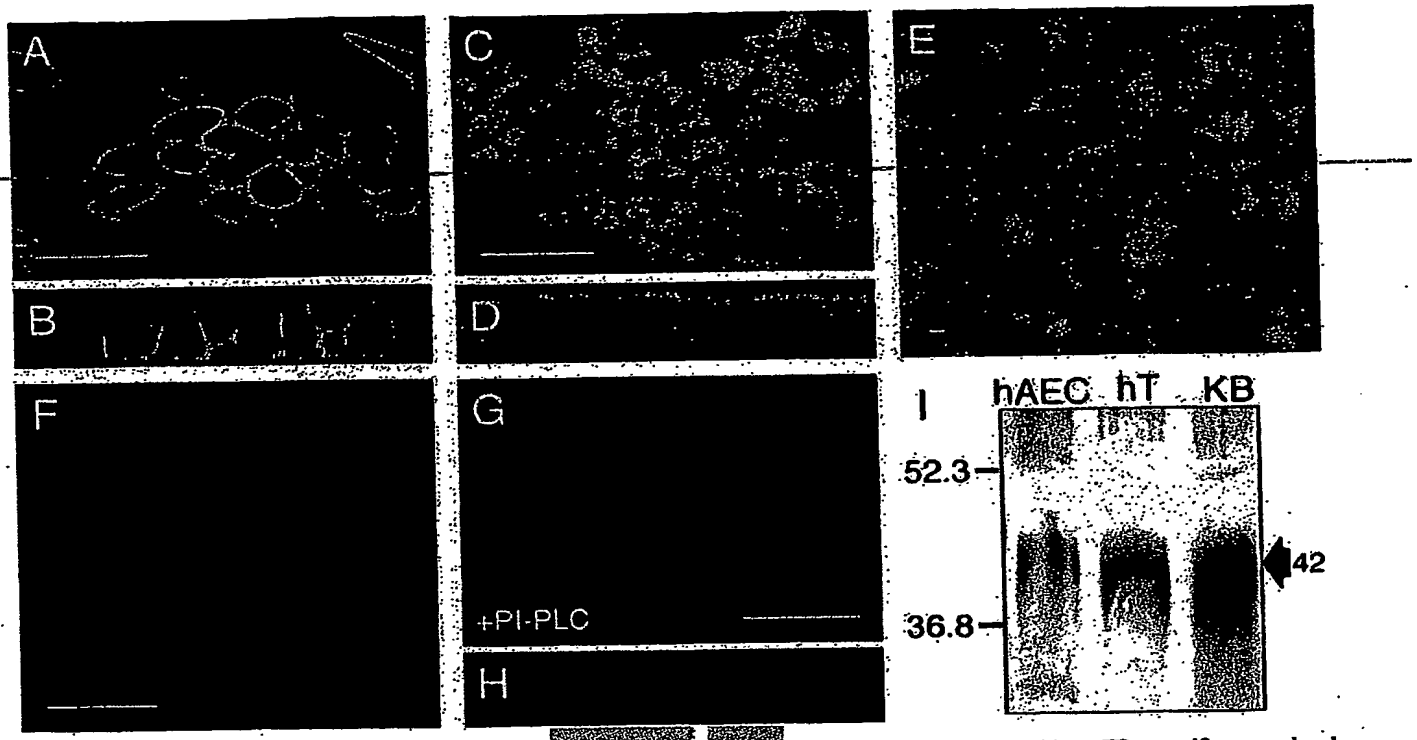

FIG. 1. FRα expression in primary cultures of human airway epithelia. Cells were fixed and incubated with an FRα-specific monoclonal antibody, followed by addition of an anti-mouse FITC-conjugated secondary antibody. KB cells were viewed by using confocal microscopy from an en face (A) or vertical section (B). Primary cultures of airway epithelia were also viewed en face at high power (C) and vertical section (D), as well as en face at low power (E). To confirm antibody specificity, an isotype control primary antibody was used (F). Primary cultures of human airway cells were also imaged following PI-PLC treatment to confirm enzyme function (en face [G] and vertical section [H]). Western blot of indicated protein samples was conducted by using the same FRα-specific monoclonal antibody followed by an anti-mouse alkaline phosphatase-conjugated secondary antibody. The expected 42-kDa band is indicated with an arrow. hAEC, human airway epithelial cell; hT, human trachea; KB, KB cell line. Scale bars = 50 μm (A, C, F, and G) or 100 μm (E).

We investigated FRα expression in primary cultures of well-differentiated human airway epithelia. To determine the polarity of expression, we immunostained the primary cultures with an FRα-specific monoclonal antibody under nonpermeabilizing conditions and imaged the cells with confocal microscopy. KB, a cell line known to express FRα at high levels, exhibited abundant cell surface levels of FRα (Fig. 1A) with no polarity of expression when viewed in vertical sections (Fig. 1B). Similarly, FRα protein expression was easily detected by immunostaining primary cultures of airway epithelia (Fig. 1C). When viewed in vertical sections, FRα was abundantly expressed at the apical surface (Fig. 1D). Interestingly, when viewed en face at a lower magnification, the distribution of FRα was heterogeneous (Fig. 1E). The reason for this expression pattern is not yet known; however, initial observations suggest that the pattern is not the result of cell-type-specific expression (e.g., ciliated versus nonciliated cells). Furthermore, the distribution was not affected by culturing cells under folate-free or excess-folate conditions (data not shown). No fluorescent signal was detected when an IgG1 isotype control primary antibody and the FITC-conjugated secondary antibody were used (Fig. 1F). As an added control to verify antibody specificity, the epithelia were pretreated with an enzyme that cleaves GPI linkages, i.e., PI-PLC. As shown, PI-PLC pretreatment removed detectable FRα expression as evaluated en face (Fig. 1G) or in vertical sections (Fig. 1H). Further confirmation of FRα expression was achieved by the detection of a 42-kDa band by Western blot analysis of primary airway cells, human trachea, and KB cell lysates (Fig. 1I). These data demonstrate that, in a polarized sheet of primary epithelia at a given time, not all cells express FRα but that within FRα-positive cells there is substantial expression at the apical surface.

Pseudotyping FIV-based vectors with filoviral glycoproteins. Abundant filovirus receptor is localized at the apical surface of airway epithelia; therefore, we hypothesized that pseudotyping FIV vector with filoviral glycoproteins would confer apical transduction properties. High viral titers facilitate in vitro experiments and are of prime concern when one is designing in vivo experiments. We routinely attain titers ranging from 10^8 to 10^9 TU/ml by pseudotyping FIV-based vectors with the MuLV amphotropic, VSV, or Ross River virus envelope glycoproteins following a 250-fold centrifuge concentration (12, 26, 28, 29). However, pseudotyping FIV-based vectors with filoviral envelope glycoproteins resulted in significantly lower viral titers. As shown in Table 1, when FIV was pseudotyped with the wild-type EBO and MRB glycoproteins, we achieved average titers of 5.5×10^6 TU/ml and 2.5×10^4 TU/ml, respectively, follow-

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TABLE 1. Modifications of the MRB and EBO envelope glycoproteins and the resultant titers of pseudotyped FIV vectors[a]

Construct name	Description of mutation	Increase (*n*-fold)	Mean ± SE	*n*
EBO WT			$5.5 \times 10^6 \pm 3 \times 10^5$	8
EBOΔO	Deletion of EBO amino acids 309–489 inclusive	73.98	$4.1 \times 10^8 \pm 1 \times 10^8$	10
MRB WT			$2.5 \times 10^6 \pm 8 \times 10^5$	6
MRBΔO	Deletion of MRB amino acids 294–424 inclusive	0.01	$2.5 \times 10^2 \pm 2 \times 10^2$	4
MRB C671A	Cysteine-to-alanine mutation at position 671	2.44	$6.0 \times 10^6 \pm 2 \times 10^6$	7
MRB C671S	Cysteine-to-serine mutation at position 671	0.58	$1.4 \times 10^6 \pm 9 \times 10^5$	6
MRB C673A	Cysteine-to-alanine mutation at position 673	1.18	$2.9 \times 10^6 \pm 1 \times 10^6$	4
MRB C673S	Cysteine-to-serine mutation at position 673	0.16	$3.8 \times 10^5 \pm 1 \times 10^5$	4
MRB F676stop	Phenylalanine to stop codon at position 676; isoleucine to lysine at position 675	2.43	$6.0 \times 10^6 \pm 5 \times 10^6$	4
MRB Y679stop	Tyrosine to stop codon at position 679	1.00	$2.5 \times 10^6 \pm 2 \times 10^6$	6

[a] Construct names and the descriptions of the mutations are indicated. WT, wild type. Titers are expressed as the mean (TU per milliliter) plus or minus standard error. The increases (*n*-fold) were calculated by normalizing the corresponding titer of FIV vector pseudotyped with the wild-type glycoprotein to 1. Significant increases of mutant glycoprotein pseudotyped FIV vector titer over that of wild-type counterparts are indicated in boldface type.

ing a 250-fold centrifuge concentration. We engineered alterations in the envelope constructs designed to enhance filoviral glycoprotein incorporation into FIV virions and tested the effects on viral titer.

Deletion of the O-glycosylated region from the extracellular domain of filoviral glycoproteins. An initial strategy for enhancing filovirus-pseudotyped FIV vector titer was to delete an expansive region from the extracellular domain thought to be heavily O glycosylated. By deletion of this region, the efficiency of envelope protein synthesis and of transport to the cell surface is enhanced (9). This region may be functionally less important than the flanking regions of the protein [illegible] cause there is little sequence conservation in this region among all filoviral isolates. The deletion of amino acids 309 to 489 from the EBO glycoprotein (EBOΔO) resulted in a marked 74-fold increase in titer over the average titer obtained with the wild-type EBO glycoprotein (Table 1). Unfortunately, a comparable deletion in the extracellular domain of the MRB construct (MRBΔO) resulted in a dramatic loss of titer. Since potential differences between the EBO and MRB pseudotype transduction efficiencies were discovered to be of interest, multiple additional avenues were therefore pursued to increase MRB viral titer.

Mutating cytoplasmic tail acylation sites or generating cytoplasmic tail truncations of the MRB envelope glycoprotein. Multiple studies have demonstrated that pseudotyping efficiency is influenced by the nature of the glycoprotein cytoplasmic domain (3, 16, 33). We designed alterations to the MRB envelope glycoprotein cytoplasmic domain intended to relieve steric interference or alter protein folding in such a way as to promote glycoprotein incorporation into the assembling virion. The MRB envelope glycoprotein contains two intracellular, potentially acylated cysteines that may interfere with efficient virion assembly. Each cysteine was mutated to either an alanine or a serine (Table 1). Encouragingly, the C671A mutation resulted in a greater-than-twofold increase in viral titer; however, the other point mutations resulted in no titer enhancement. The incorporation of a serine at either position significantly decreased viral titer (Table 1). In addition to these point mutations, we constructed two C-terminal deletions of the MRB envelope glycoprotein. Deleting the terminal 3 amino acids (Y679stop) had no effect on FIV vector titer compared to that of the wild-type glycoprotein; however, deleting the terminal 6 amino acids (F676stop) resulted in a greater-than-twofold increase in viral titer (Table 1). The later construct introduces a lysine at position 675 for proper anchoring of the glycoprotein in the plasma membrane. Although the enhancements in titer were modest, these data demonstrate the potential of C-terminal mutagenesis of the MRB envelope glycoprotein to boost vector titer.

Replacing the cytoplasmic tail of the MRB envelope glycoprotein with the MuLV amphotropic or FIV envelope cytoplasmic tail. Replacing the C terminus of the MRB envelope glycoprotein with that of another glycoprotein known to efficiently incorporate into budding FIV virions is an additional strategy that we pursued to enhance the viral titers with the MRB glycoprotein. The amphotropic (ampho) envelope glycoprotein from MuLV was a prime candidate for chimera construction for multiple reasons. Importantly, both the MRB and ampho glycoproteins are type 1 transmembrane proteins that form homotrimers when expressed on the cell surface (17, 30). In addition, similar strategies have proven effective for pseudotyping lentivirus vectors (21, 24). Using biochemical analyses and sequence homologies of the MRB and ampho glycoproteins (20), we chose to engineer the fusion site at MRB glycoprotein residue 670 and ampho 619 (termed MRB/ampho). In addition, we fused the MRB extracellular and transmembrane domains to an ampho intracellular domain with a mutation in the putative endocytosis signal (termed MRB/amphoY665A) (8) or a truncated ampho C terminus (termed MRB/amphoΔ650/675). Unfortunately, none of the MRB/ampho chimeric glycoproteins enhanced vector titers (data not shown).

In addition to MRB/ampho chimeric glycoproteins, we pursued a parallel approach by using the native FIV envelope glycoprotein sequence to generate MRB/FIVenv chimeric glycoproteins. We fused the MRB extracellular domain and transmembrane domain to the native FIV envelope intracellular domain. We hypothesized that the native C terminus of the envelope protein sequence would efficiently incorporate into the assembling vector. The chimera junction point is likely critical; therefore, we chose multiple fusion points ranging from amino acids 807 to 815 of the FIV envelope. However, none of the MRB/FIVenv chimeric constructs resulted in an increase of FIV vector titer (data not shown).

In summary, of the MRB glycoprotein mutations, only

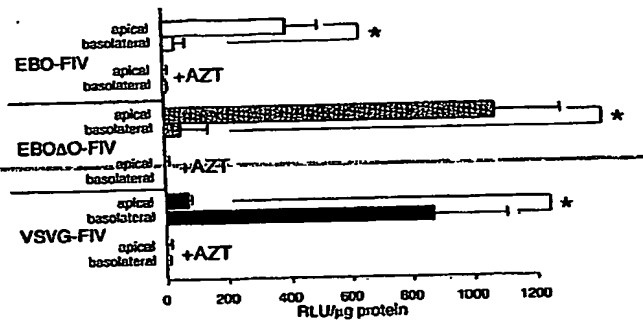

FIG. 2. Transduction levels of polarized airway cell lines with pseudotyped FIV vector. Primary cultures were transduced with pseudotyped FIV vector applied to the apical or basolateral surface. In addition, as a control for pseudotransduction, cells were pretreated with AZT for 24 h before vector application. Four days after initial vector incubation, cells were harvested and the β-galactosidase activity was quantified and normalized to total protein. $n = 3$ (samples from three independent human specimens). RLU, relative light units. *, $P < 0.01$.

C671A and F676stop resulted in increased FIV vector titers (Table 1). However, these increases were modest and did not confer the titers conducive to multifaceted in vitro experiments with MOIs greater than 1. For this reason, the subsequent [illegible] studies focused on vectors pseudotyped with the [illegible] EBOΔO glycoproteins.

Apical transduction of human airway epithelia by filovirus-pseudotyped FIV. To test the polarity of vector transduction in primary cultures of human airway epithelia, FIV pseudotyped with wild-type EBO glycoprotein (EBO-FIV), EBO envelope protein with the deletion of positions 309 to 489 (EBOΔO-FIV), or VSV glycoprotein VSVG-FIV was applied to the apical or basolateral surface as indicated for 4 h at an MOI of ~5 (Fig. 2). Following vector application, the cells were washed and incubated for 4 days before quantification of β-galactosidase expression as described in Materials and Methods. Both EBO-FIV and EBOΔO-FIV transduced airway epithelia from the apical surface at greater efficiency than from the basolateral surface. In contrast, VSVG-FIV transduced the basolateral surface more efficiently than the apical surface. In each case, pretreating the epithelia with AZT abolished β-galactosidase expression, indicating that the observed β-galactosidase activity is not the result of pseudotransduction. These data indicate that filovirus-pseudotyped FIV vectors preferentially transduce airway epithelia from the apical surface, thus providing indirect evidence in support of FRα as a receptor for vector entry.

Blocking transduction of filovirus-pseudotyped FIV with FRα inhibitors. To examine the role of FRα as a receptor for filovirus-pseudotyped FIV, FRα-blocking or -cleaving experiments were conducted on primary cultures of well-differentiated human airway epithelia (Fig. 3). In each condition, FRα-specific blocking antibody or an isotype control antibody was applied to both the apical and basolateral surfaces of the airway epithelia (Fig. 3A). Following the incubation with the antibody, the vector was administered to the apical or basolateral surface as indicated (Fig. 3A). The ability of the viral vector to transduce cells was quantified by β-galactosidase activity normalized to total protein. The FRα-blocking antibody had no effect on the VSVG-FIV control vector. Contrary to expectation, application of the FRα-blocking antibody had no effect on transduction efficacy when EBOΔO-FIV was applied to either the apical or basolateral surface compared to the isotype antibody (Fig. 3A). Apical transduction of EBOΔO-FIV remained significantly higher than basolateral transduction in the presence or absence of the blocking antibody.

To complement the FRα-blocking antibody studies, we pursued additional experiments utilizing the GPI-linkage cleaving enzyme, PI-PLC, to remove FRα from the cell surface. The ability of PI-PLC to cleave FRα from primary cells was evaluated by immunofluorescence (Fig. 1G and H). Cells were pretreated with PI-PLC, followed by incubation with EBOΔO-FIV or VSVG-FIV. Similar to the blocking antibody, PI-PLC did not reduce the transduction efficiency of EBOΔO-FIV or the VSVG-FIV control vector in primary cultures of airway epithelia (Fig. 3B). Again, apical transduction of EBOΔO-FIV remained significantly higher than basolateral transduction in the presence or absence of PI-PLC. These data indicate that FRα is not required as a receptor for EBOΔO-FIV in primary cultures of human airway epithelia.

Expression of FRα in immortalized airway epithelial cells. In light of this unexpected observation, we evaluated the potential role of FRα as a mediator of filoviral entry into multi[illegible] immortalized cell lines. We quantified the levels of FRα mRNA (Fig. 4A) and FRα protein (Fig. 4B) in control cell lines and airway epithelium-derived cell lines. KB is perhaps the most commonly utilized cell line for studying FRα in vitro

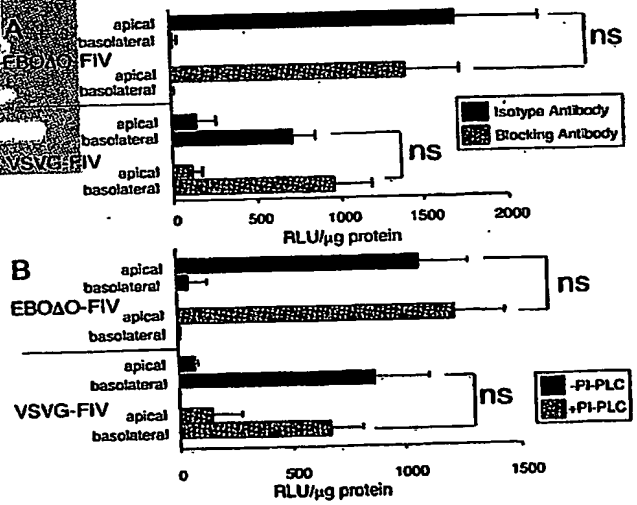

FIG. 3. Transduction levels of polarized airway cell lines with pseudotyped FIV vector following FRα-blocking antibody or PI-PLC treatment. (A) Following pretreatment with an IgG1 isotype antibody (black bars) or an FRα-blocking antibody (gray bars), primary cultures were transduced with pseudotyped FIV vector applied to the apical or basolateral surface. (B) Following pretreatment with PI-PLC (gray bars) or without PI-PLC (black bars), primary cultures were transduced with pseudotyped FIV vector applied to the apical or basolateral surface at an MOI of 5. Four days after initial vector incubation, cells were harvested, analyzed by β-galactosidase assay, and normalized to total protein. $n = 3$ (samples from three independent human specimens). RLU, relative light units. *, $P < 0.01$.

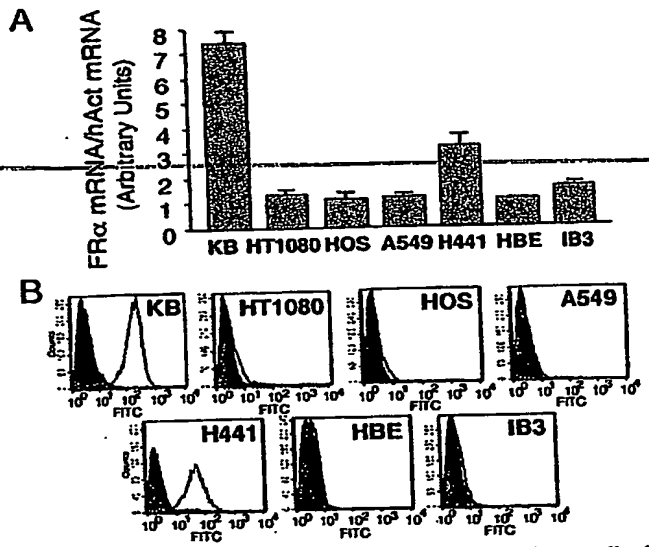

FIG. 4. Relative FRα mRNA and protein levels from immortalized cell lines. (A) Total mRNA from the indicated cell lines was purified and analyzed by RPA by using FRα and human actin (hACT)-specific [α^{32}P]UTP-labeled antisense probes. Signal abundance was quantified with a PhosphorImager, and FRα expression was normalized to actin expression. $n = 3$. (B) To determine relative FRα protein abundance, protein lysates from the indicated cell lines were incubated with either an FRα-specific monoclonal antibody (open curve) or an isotype control (shaded curve). Samples were then incubated with an anti-mouse FITC-conjugated secondary antibody and were subjected to FACS analysis as described in Materials and Methods. $n \geq 2$.

and therefore, as expected, displayed abundant FRα mRNA and protein. Interestingly, our FIV vector-titering cell line, HT1080, exhibited minimal expression of FRα mRNA and protein (Fig. 4). Two cell lines utilized by Chan and colleagues (2), HeLa (not shown) and HOS, displayed relatively high and low FRα levels, respectively. Of the four airway-derived immortalized cell lines tested (H441, HBE, A549, and IB3) only H441 exhibited relatively high levels of FRα by either RPA or FACS analysis.

Transduction of immortalized airway epithelia by filovirus-pseudotyped FIV. The indicated cell lines were transduced at an MOI of ~5 with EBO-FIV, EBOΔO-FIV, or VSVG-FIV (Fig. 5A). Due to titer limitations, the cell lines were transduced with wild-type MRB envelope-pseudotyped FIV (MRB-FIV) at an MOI of ~0.5 (Fig. 5B). As shown, HT1080 cells were consistently transduced with the greatest efficiency for all vectors (Fig. 5A and B) despite expressing only low levels of FRα (Fig. 4). KB cells and H441 cells expressed much higher levels of FRα than HT1080 cells but transduced at ~50% the efficiency of HT1080 cells. IB3, HBE, HOS, and A549 were transduced at lower levels. EBO-FIV and EBOΔO-FIV transduced each cell line with similar efficiency, and the pattern of transduction between the cell lines was closely comparable with that of the MRB-FIV. Interestingly, the transduction efficiency of the VSVG-FIV was not significantly different from those of the EBO-FIV and EBOΔO-FIV except for the A549 cell line. VSVG-FIV transduced A549 cells at approximately fivefold-greater efficacy than EBO-FIV or EBOΔO-FIV. F5

Blocking transduction of filovirus-pseudotyped FIV with FRα inhibitors. The contribution of FRα in facilitating filovirus-pseudotyped FIV binding and entry into airway-derived and control cell lines was further tested by pretreating cells with an FRα-specific blocking antibody or an isotype control antibody (Fig. 6). The ability of the viral vector to transduce cells was again quantified by β-galactosidase activity normalized to total protein. The transduction of KB and HT1080 cells was not significantly affected by pretreatment with the blocking antibody. These data suggest the existence of FRα-independent pathways for filoviral infection. Of the airway-derived cell lines, the blocking antibody successfully reduced transduction of the EBO-FIV and EBOΔO-FIV in H441 and IB3 cells but not in A549 or HBE cells. As previously observed by Chan et al., the FRα-blocking antibody successfully reduced the transduction efficiency in HOS cells (2). Importantly, EBO-FIV and EBOΔO-FIV were blocked to a similar extent in each cell line, suggesting that deleting the O-glycosylated region of the EBO glycoprotein does not alter its binding and fusion specificity or cell tropism. F6

In addition, each cell line was transduced with VSVG-FIV in

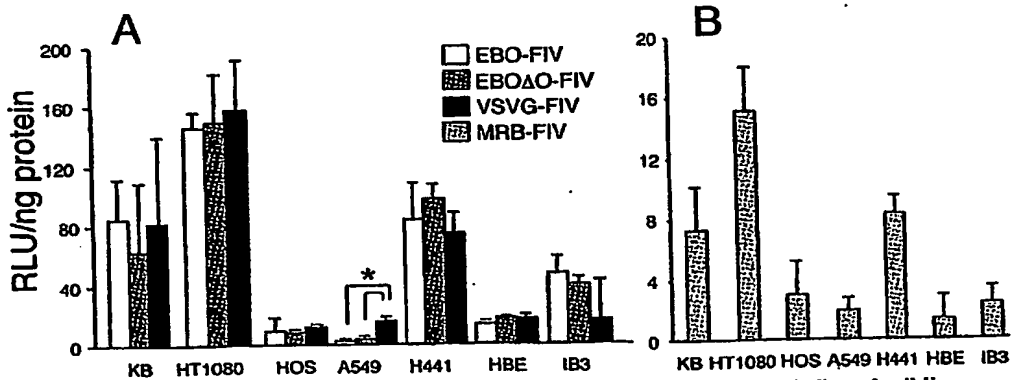

FIG. 5. Relative transduction levels of immortalized cell lines with pseudotyped FIV vector. The indicated cell lines were transduced with EBO-FIV, EBOΔO-FIV, or VSVG-FIV at an MOI of 5 (A) or MRB-FIV at an MOI of 0.5 (B). Four days after initial vector incubation, cells were harvested, analyzed by β-galactosidase assay, and normalized to total protein. MOIs were calculated by using vector titers determined on HT1080 cells. RLU, relative light units. $n = 3$. *, $P < 0.01$.

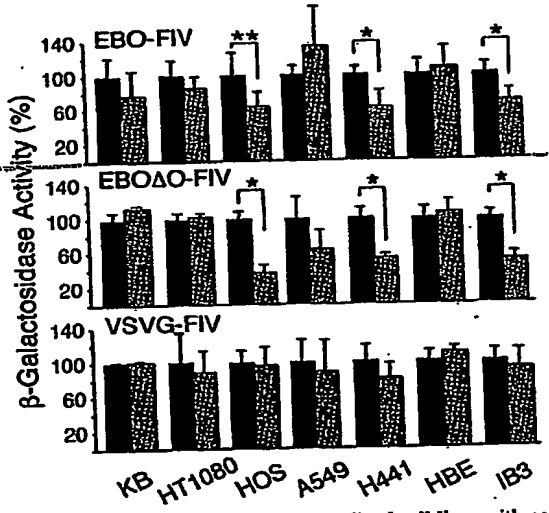

FIG. 6. Transduction levels of immortalized cell lines with pseudotyped FIV vector following FRα-blocking antibody treatment. Following pretreatment with an IgG1 isotype antibody (black bars) or an FRα-blocking antibody (gray bars), the indicated cell lines were transduced with EBO-FIV, EBOΔO-FIV, or VSVG-FIV at an MOI of 5. Four days after initial vector incubation, cells were harvested, analyzed by β-galactosidase assay, and normalized to total protein. The mean β-galactosidase activity following isotype antibody pretreatment for each cell line and vector administration is normalized to 100%. *, $P < 0.01$; **, $P < 0.05$.

face than when applied to the basolateral surface. This notable observation is unique among the many pseudotyped retroviral vectors reported to date and is consistent with previously published results (14). Kobinger et al. (14) demonstrated that EBO-pseudotyped human immunodeficiency virus transduced the apical surface of airway epithelia with greater efficacy than it did the basolateral surface. We demonstrated that the EBO envelope glycoprotein confers its apical transducing ability to a nonprimate lentiviral vector and tested its ability to utilize FRα as an avenue for cellular entry. Interestingly, we observed abundant expression of FRα at the apical surface by immunohistochemistry (Fig. 1). Indeed, GPI-linked proteins will typically sort to the apical surface of polarized epithelial cells (15). Based on this circumstantial evidence, one might expect FRα to contribute to binding and entry of filovirus-pseudotyped FIV vectors into primary cultures of human airway epithelia; however, we found that the folate-blocking antibody or PI-PLC treatments failed to inhibit the transduction of these cells.

As summarized in Table 2, we observed various levels of T2 FRα expression in the cell lines tested. Furthermore, the relative levels of FRα protein or mRNA did not necessarily correlate with EBOΔO-FIV transduction efficiency and blocking FRα failed to inhibit transduction. Generally, we observed that PI-PLC or an FRα-blocking antibody was likeliest to perturb transduction in cell lines that already had low levels of transduction and low levels of FRα expression, such as with HOS or [illegible] cells (Table 2). However, in no cell line did the blocking efforts reduce the transduction efficiencies by greater than two-fold. Cell lines with ample levels of FRα or cells that were easily transduced with EBOΔO-FIV, such as KB and HT1080 cells, respectively, were typically not responsive to PI-PLC or blocking antibody treatments (Table 2).

One interesting outcome of our studies was the observation that the deletion of the O-glycosylation region from the EBO glycoprotein greatly increased the titers of FIV vector relative to results for the wild-type glycoprotein. In contrast, no titer

the presence or absence of the FRα-blocking antibody (Fig. 6). No inhibitory effects on VSVG-FIV transduction were found for any cell line. In cells pretreated with AZT or lamivudine (not shown), β-galactosidase activity was dramatically reduced, indicating that the observed expression is not the result of pseudotransduction.

In a manner similar to that for the experiments in the primary cultures of airway epithelia, we utilized PI-PLC to remove FRα from the cell surface of the immortalized cell lines. The ability of PI-PLC to cleave FRα from immortalized cell lines was confirmed by FACS analysis (data not shown). In each case the enzyme treatment efficiently removed FRα. Cells were pretreated with PI-PLC, followed by incubation with EBOΔO-FIV or VSVG-FIV. PI-PLC treatment did not inhibit transduction of EBOΔO-FIV in KB, HT1080, A549, or HBE F7 cells (Fig. 7). However, PI-PLC treatment did reduce the transduction efficiency of EBOΔO-FIV in HOS, H441, and IB3 cells (Fig. 7). In no cell line was the transduction efficiency of VSVG-FIV affected by pretreatment of PI-PLC. Together with the blocking antibody studies, these data further support the existence of FRα-independent entry pathways for filovirus pseudotypes in human airway epithelia (Fig. 3 and 6).

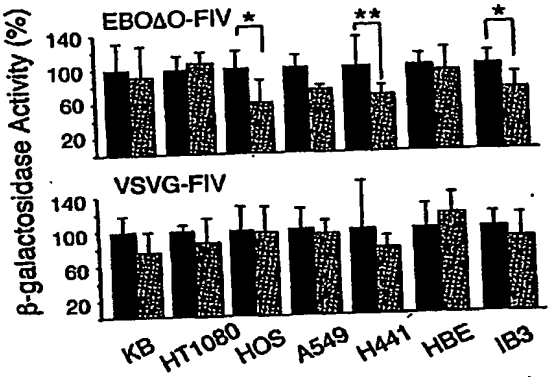

FIG. 7. Transduction levels of airway- and non-airway-derived cell lines with pseudotyped FIV vector following PI-PLC treatment. Following pretreatment with PI-PLC (gray bars) or without PI-PLC (black bars), the indicated cell lines were transduced with EBOΔO-FIV or VSVG-FIV at an MOI of 5. Four days after initial vector incubation, cells were harvested, analyzed by β-galactosidase assay, and normalized to total protein. The mean β-galactosidase activity without PI-PLC pretreatment for each cell line and vector administration is normalized to 100%. *, $P < 0.01$; **, $P < 0.05$.

DISCUSSION

In this study we investigated the contribution of FRα to the transduction ability of a filovirus-pseudotyped FIV-based vector in airway epithelia and cell lines. Importantly, EBO-FIV and EBOΔO-FIV transduced well-differentiated polarized airway epithelia more efficiently when applied to the apical sur-

TABLE 2. Summary of FRα expression and EBOΔO transduction levels of airway- and non-airway-derived cell lines[a]

Cell type	Tissue origin*	Detection assay result for:		Relative FRα level of:		Blockage by:	
		Galactolytes	Cell count	Protein	mRNA	PI-PLC	Blocking antibody
HT1080	Fibroblast	+++	+++	Low	Low	No	No
HOS	Bone	+	NT	Low	Low	Yes	Yes
KB	Cervix	++	NT	High	High	No	No
H441	Airway	++	+	High	High	Yes	Yes
HBE	Airway	+	±	Low	Low	No	No
IB3	Airway	+	±	Low	Low	Yes	Yes
A549	Airway	+	±	Low	Low	No	No
HAE	Airway	+	±	High**	Detected†	No	No

[a] Data from Fig. 1, 2, and 3 to 8 are summarized for convenience. *, all cell lines are human derived. **, data acquired by immunofluorescence and Western blotting. †, data acquired by nonquantitative reverse transcriptase PCR. HAE, primary cultures of human airway epithelia. Plus signs represent qualitative comparisons as follows: +++, very abundant expression, ++, moderate expression; +, detectable expression; ± expression detectable at limit of assay resolution; and NT, not tested.

benefit was observed in previous studies in which Simmons et al. (22) constructed EBO O-glycosylation deletion mutants. Therefore, the deletion site is likely critical. Biochemical analysis of the EBOΔO construct by Jeffers et al. (9) indicated that O-glycosylation deletion facilitates glycoprotein processing, incorporation into retrovirus particles, and viral transduction (9). Moreover, there may be an important therapeutic benefit of deleting the putative O-glycosylation domain; the serine-threonine-rich O-glycosylated region (or mucin-like domain) has been implicated as a pathogenic determinant of EBO (32). Yang and colleagues (32) observed that this region of the glycoprotein was required for vascular cytotoxicity and concluded that it may contribute to hemorrhage during an EBO infection. In addition, Simmons et al. (22) observed that the O-glycosylated region was necessary to induce loss of cell adherence. Therefore, by constructing the EBOΔO construct for pseudotyping, we may have added a safety benefit as well as achieving a dramatic boost in titer.

Interestingly, a similar deletion of the O-glycosylation region of the MRB glycoprotein did not yield a similar increase in titer. Clearly, the selection of which amino acids to delete in such an experiment is vital to producing a functional protein; therefore, more MRB deletion constructs will need to be tested. One potential untested possibility is that the mutations resulted in increased MRB envelope production but decreased the stability of the envelope complex, leading to decreased titer following concentration by centrifugation. As a whole, our efforts to increase the titers of MRB-FIV met with only limited success. Results similar to those for EBO-FIV and EBOΔO-FIV were evident; we observed that MRB-FIV transduces the apical surface of human airway epithelia with greater efficacy than it does the basolateral surface (data not shown); therefore, examining the differences in tropism and transduction efficiencies between EBO-FIV and MRB-FIV at equivalent MOIs is of interest.

We envision a model in which EBO-FIV has multiple avenues for binding and entry into different cell types. For example, C-type lectins have recently been demonstrated to confer enhanced cellular entry efficacy for EBO-pseudotyped retrovirus in hematopoietic cells (1). The contribution of DC-SIGN and L-SIGN in airway epithelial cells has not yet been investigated; however, the study conducted by Alvarez et al. (1) supports a model of cellular entry for EBO-FIV that is more complex than a single receptor. Cell lines such as HT1080 or KB may present a number of opportunities for EBO-FIV entry. In such cells, blocking FRα has little effect on transduction levels. Conversely, a cell line such as HOS or IB3 may offer fewer pathways for EBO-FIV entry; therefore, the individual contribution of FRα is much greater. Our data confirm the previous finding that the FRα acts as a cofactor contributing to filovirus entry into some but not all cell types (2).

In conclusion, our results do not eliminate the possibility that FRα may contribute to the binding and entry of filovirus-pseudotyped lentivirus into airway epithelia in vivo; however, its presence was not required to achieve transduction in primary cultures. These data also indicate that other unknown cellular factors are functioning as viral receptors for filovirus-pseudotyped lentivirus. These results confirm that filoviral glycoproteins are excellent candidates for pseudotyping lentiviral vectors to target the apical surface of airway epithelia. Although further challenges must be overcome for CF gene therapy, the availability of an integrating vector that transduces polarized airway epithelia cells from the apical surface will facilitate additional preclinical studies in vitro and in vivo.

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